ROYAL
BOTANIC GARDENS
VICTORIA

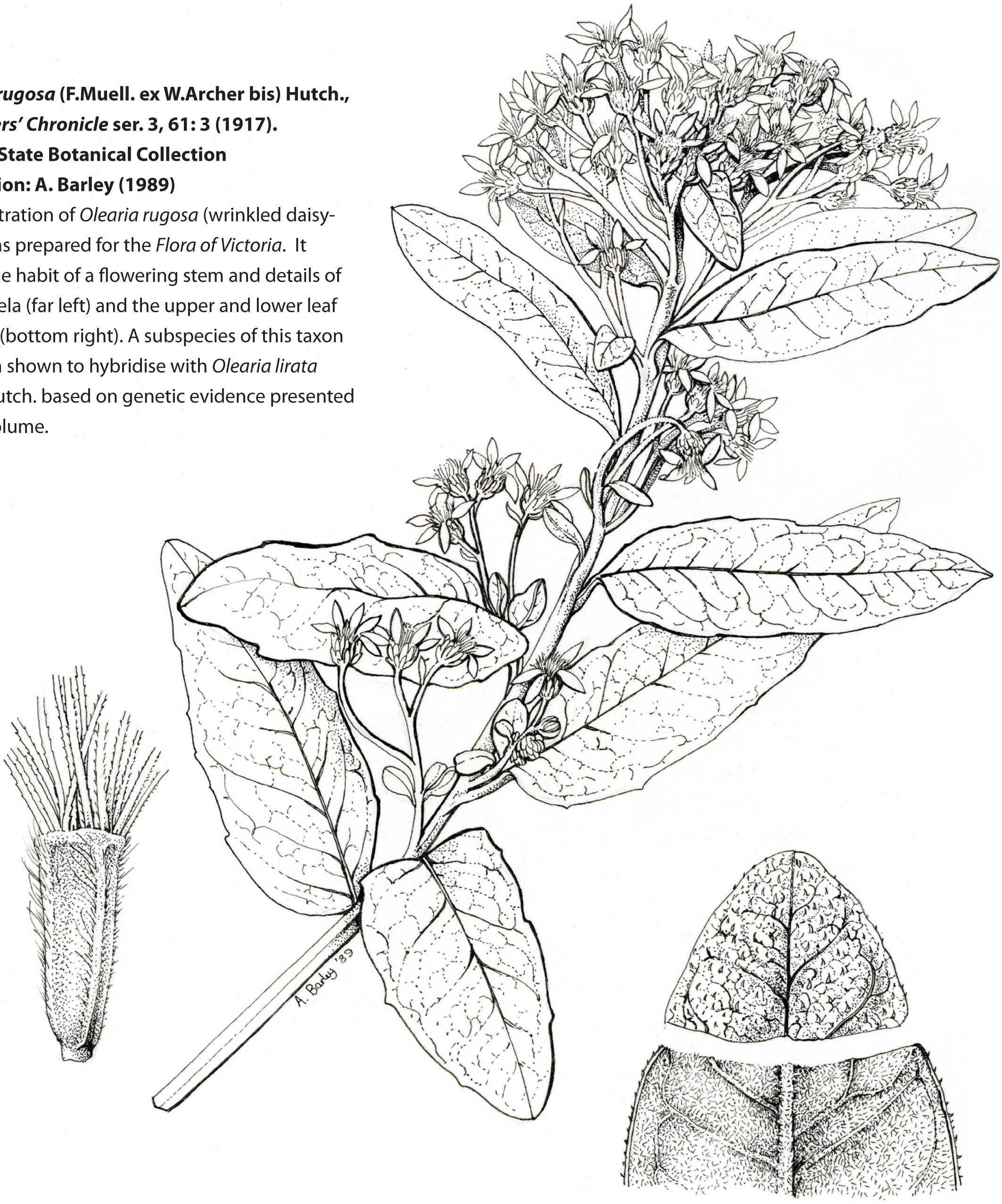
# Muelleria

Plant, Algal and Fungal Taxonomy and Systematics

Vol 43 Genomics Special Issue, 2025

***Olearia rugosa*** **(F.Muell. ex W.Archer bis) Hutch.,**
***Gardeners' Chronicle*** **ser. 3, 61: 3 (1917).**
**Source: State Botanical Collection**
**Illustration: A. Barley (1989)**

This illustration of *Olearia rugosa* (wrinkled daisy-bush) was prepared for the *Flora of Victoria*. It shows the habit of a flowering stem and details of the cypsela (far left) and the upper and lower leaf surfaces (bottom right). A subspecies of this taxon has been shown to hybridise with *Olearia lirata* (Sims) Hutch. based on genetic evidence presented in this volume.



*Muelleria* publishes research papers on Southern Hemisphere plant, algal and fungal taxonomy and systematics, particularly works relating to Australia. Acceptable submissions include: taxonomic revisions; phylogenetic and biogeographical studies; short papers describing new taxa (including global taxa of genera represented in the indigenous flora of Australia), documentation of nationally significant new records, or resolutions of nomenclatural matters; historical analyses relevant to systematics; and any research contributing to our knowledge of plant, algal or fungal diversity. Special issues or reviews will be considered after discussion with the Editor.

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# Muelleria

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# From Mueller to the genomics era: plant conservation at Royal Botanic Gardens Victoria

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Since its founding in the 19th century through to the present day, the story of plant conservation at Royal Botanic Gardens Victoria (RBGV) and the National Herbarium of Victoria has been one of continuity, innovation and restoration. Two centuries after the birth of Ferdinand von Mueller, the first Director of the Royal Botanic Gardens Melbourne, his legacy of scientifically rigorous and country-wide connected botanical research endures in the institution he shaped, and in the collections and community he fostered. Although the challenges have shifted – from cataloguing and describing a largely unknown flora to understanding how best to protect native plants in the face of growing environmental pressures – the role of the botanic gardens in conservation remains a defining constant.

Born in Rostock, Germany, in 1825, Ferdinand Mueller was destined for an illustrious career far from his country of birth. In 1847 he migrated to Australia, initially settling in South Australia before moving to Melbourne in 1851. In 1853 he was appointed Government Botanist, a position he held until his death in 1896. He founded the National Herbarium of Victoria, building a world-class herbarium collection that today is a key State asset for species discovery and plant conservation research. In 1857 he became the first Director of the Royal Botanic Gardens Melbourne. His tenure as Director lasted until 1873, during which time he built a diverse collection of flora from around the world. In 1862 he recorded that the Gardens sent out 42,519 seed packets to public institutions in Victoria and 28,589 interstate (Mueller 1862), illustrating the importance of Melbourne as a centre of plant exchange. More than that, the distribution of seeds around the world highlights the formidable network of people that Mueller built and who contributed to both the herbarium and the Gardens. His diverse collection of living species was inherited by William Guilfoyle and transformed into the gardens we see today.

During Mueller's lifetime he took part in, and was responsible for, several European-led expeditions through Australia, including the Augustus Gregory expedition across northern Australia, where he collected 800 species new to western science (Morris 1974). It is estimated that, over his lifetime, Mueller collected more than 25,000 specimens and coined nearly 8,000 names for Australian plants of which more than 1,500 remain in use and are accepted by botanists as valid concepts today (representing approximately 7% of the Australian flora).

Mueller was not unaware of the impact that colonisation of Australia was having on its native vegetation through his role as a commissioner on the Royal Commission on Foreign Industries and Forests. In a lecture on 'Forest culture in its relation to industrial pursuits', he identified "the forest as an heritage given to us by nature, not for spoil or to devastate, but to be wisely used, reverently honoured, and carefully maintained" (Mueller 1871). One hundred and twenty-four years later, we face a crisis: clearing and fragmentation of ecosystems, introduced animals and plants, and environmental change are pushing native flora and fauna towards extinction. It is a repeatedly stated fact that Australia has the worst record for mammal extinctions on the planet, with 39 species listed as extinct (EPBC Act 1999), but what about plants? An examination of the *Environment Protection and Biodiversity Conservation Act 1999* threatened species list reveals 1,490 threatened plant species, compared to 685 animals. Thirty-five plant species are listed as extinct, including species described by Mueller (e.g. *Musa fitzalanii*, the Daintree river banana), but many more (289 species) are critically endangered.

While plants are disproportionately represented on state and federal threatened lists, actions to conserve them are limited when compared to the investment directed toward animals. Yet without plants there is no life on Earth. Plants produce the oxygen we breathe, the food we eat, provide medicines that heal us, and fibres that clothe us. However, as a society we suffer from what has been termed "plant blindness" (Wandersee and Schussler 1999), an inability to notice and appreciate plants in our surroundings. More correctly, this condition should be called "everything-but-vertebrates bias" (Knapp 2019). Although we recognise the importance of plants, the innate bias towards vertebrates flows through to conservation decisions, where arguably more resources are spent on singular vertebrate species (e.g. the koala) than are spent on all plant species combined, threatened or not. Without investment in plant conservation, our ability to conserve the animals that rely on the vegetation will fail. It has been estimated that we need to increase investment in conservation in Australia by a factor of seven to achieve any meaningful species recovery (Wintle *et al*. 2019).

In 2025, we celebrate the 200th anniversary of Mueller's birth with a special issue on plant conservation genetics. The 2019/2020 Black Summer megafires in Australia burnt more than 8 million hectares of vegetation in the country's south-east alone. These fires affected a wide range of vegetation communities, including rainforests, eucalypt forests and woodlands, as well as shrublands and heathlands, with more than 800 vascular plant species having 50% or more of their populations or ranges burnt (Godfree *et al.* 2021). In Victoria, areas that were particularly impacted include the pockets of warm-temperate rainforest in the southeast of the state, montane grassy shrublands, ironbark forests, eucalypt and banksia woodlands, and heathlands (Geary *et al*. 2021). Management actions for the flora most at risk of extinction (i.e., species already threatened or with substantial loss of habitat) require assessment of population health to provide targeted responses specific to each species (McDonald *et al.* 2015). From a genetic perspective, understanding the patterns of genetic diversity within and between populations of a species is crucial to first evaluating the genetic health of a species and to guiding management strategies such as targeted seed collecting or genetic rescue of at-risk populations (Broadhurst *et al*. 2016). However, much of the Victorian flora has not had the fine-scale genetic analyses needed to effectively inform genetic risks that would guide management actions.

The advent of high-throughput molecular sequencing has opened up new tools for conservation and approaches that can be implemented at scale. One such example in Australia is the *Restore and Renew* program developed by the Research Centre for Ecosystem Resilience at the Royal Botanic Gardens Sydney (Rossetto *et al*. 2018). This program uses genomic data to allow restoration practitioners to make informed genetic decisions when putting plants back into the landscape, though with an NSW-specific focus. Based on this model, RGBV has obtained funding to produce similar analyses for some threatened and endangered flora of Victoria.

In this special issue of *Muelleria*, we highlight how, for a relatively small investment, sound conservation management decisions can be made using these DNA technologies. Much like in Mueller's time, delivery of these studies has relied on the researchers developing strong networks of committed land managers who are passionate about saving threatened species. We have brought together six research projects covering a range of taxa, from highly disjunct (*Acacia lucasii*; Simmons *et al*. 2025a) or range-restricted (*Grevillea jephcottii*; Hopley *et al*. 2025a) species with limited gene flow and distinct genetic signatures, to fragmented populations (*Eucalyptus mackintii*; Hopley *et al*. 2025b) with currently high levels of gene flow. Other studies, e.g. Hopley *et al*. 2025c, have documented the patterns of genetic diversity in the landscape for *Grevillea celeata*, but importantly have identified the issues created when decisions are made in the absence of genetic data. The other studies highlight the challenges associated with closely related species, how best to select translocation sites to reduce the risks of hybridisation (Simmons *et al*. 2025b), and how to respond to clonality (Simmons *et al*. 2025c).

Such studies not only provide essential baseline information for the conservation of individual species; by driving the accumulation of such fine-scale genetic data across the Victorian flora, they allow us to extrapolate and provide information to support the conservation needs of entire ecosystems.

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# Genetic rescue a viable option for the range-restricted endemic shrub *Grevillea celata* Molyneux (Proteaceae) with history of ineffective translocations

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## Abstract

Restricted geographic range is a strong indicator of a species' extinction risk and is often coupled with small, isolated populations that are susceptible to genetic decline. Genetic analysis of the critically endangered *Grevillea celata* Molyneux, restricted to an area of 60 km$^2$ in eastern Victoria, reveals that genetic connectivity diminishes with distance between its sites. One small population was found to harbour a unique genetic signature that merits preservation. One translocation site was found to be monoclonal and this location in particular would be a good candidate for genetic augmentation. Root-suckering was observed and must be considered when collecting germplasm for conservation.

***Keywords*:** Conservation, endangered, ex situ, in situ, population genomics, DArTseq

## Introduction

Recent decades have seen a push towards understanding the conservation status and threats to plant species, in large part due to the Global Strategy for Plant Conservation which had a target of assessing the conservation status of all known plant species by 2020 (Convention on Biological Diversity, 2012). This is an ongoing process with an estimated 21–26% of known plant species assessed by 2016 (Bachman *et al*. 2018), and 37–44% of assessed plant species considered threatened (Nic Lughadha *et al*. 2020). Australia has more than 18,000 endemic species, the second- highest ranked country, and just under 40% of those species had threat assessments by 2022 (Gallagher *et al*. 2023). A meta-analysis of 1,135 threatened Australian taxa found nearly 40% (418) are still declining and, of these, a quarter were ranked as being at risk of extinction under current management regimes (Silcock & Fensham 2018).

Species geographic range size includes both extent of occurrence and area of occupancy, either of which, when limited, place species at a high risk of extinction particularly when populations are severely fragmented (IUCN, 2012). Conservation of range-restricted species is challenging as they are more susceptible to threats impacting the whole

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species in one event. Larger range and large population size can safeguard a species against localised threats reducing the chance of range-wide impacts. With the increasing risk of extreme weather, particularly fires in Australia, the chances of a range-wide threat are heightened (Collins et al. 2022; Ellis et al. 2022). Within the geographic range for a species, subpopulations that are small and relatively isolated may be at greater risk of extinction (Broadhurst et al. 2017). In small and isolated subpopulations, a loss of genetic diversity may lead to an increased incidence of inbreeding and production of plants that lack vigour or are susceptible to pathogens (Keller & Waller 2002). Fragmentation reduces gene flow among subpopulations and increases the chance of local adaptation which can reduce the fitness of populations if climatic conditions change at a faster rate than species can adapt or migrate (Aitken & Whitlock, 2013). Adaptation to harsh conditions often encountered at the edge of range distributions can develop, and this local adaptation may harbour unique diversity that could support survival through future extreme predicted conditions. Too much gene flow into such marginal populations could result in outbreeding depression if it leads to disruption of local adaptations (Forrest et al. 2011). Alternatively, small, isolated populations may be genetically depauperate, lacking fitness and evolutionary potential (Ellstrand & Elam 1993).

Population genetic processes critically depend on plant species mating system and life history traits. When reproduction is asexual only, genetic diversity within populations becomes static, removing the ongoing process of adaptation via recombination that is conducive to persistence in a changing environment. Asexual reproduction, such as resprouting or root-suckering can be an adaptive mechanism for maintaining landscape persistence under temporary or ongoing conditions sub-optimal for seed production or recruitment (Vallejo-Marín *et al*. 2010). Resprouting or root-suckering in response to fire will result in pre-fire genetic diversity being maintained, alleviating the reliance on a seedbank for survival. Shorter fire intervals in the absence of resprouting could lead to local extinction if the seedbank does not have time to replenish between fires. If fire frequency increases, resprouting plants will be able to maintain levels of genetic diversity post-fire but this advantage will only last until plants senesce, while there is also the risk that repeated fires or high intensity fires may kill plants (Fairman *et al*. 2019).

Assessment of a species' extinction risk and identification of key threats inform conservation actions to mitigate threats. While some threats such as wild fires are challenging to predict and prepare for, smaller more predictable threats, such as road works, may be alleviated using a combination of methods such as *ex situ* propagule storage and translocations. Translocations may be composed of multiple different propagule sources such as seeds, whole plants, or cuttings. Most historic translocations in Australia have been performed with the intention of reducing extinction risk, however the majority have lacked guidance from genetic studies (Silcock *et al*. 2019). Commonly, many propagules are used (Silcock *et al*. 2019) but there is the risk that propagules may have been sourced from a limited number of genetically diverse plants such as cuttings, or seed taken from a single, few or closely related individuals (Broadhurst *et al*. 2021). Similarly, some *ex situ* germplasm collections have not necessarily incorporated the extent of genetic variation present in natural populations and diversity can be lost from *ex situ* collections over time (Diaz-Martin *et al*. 2023). Genetic assessment and monitoring of historic translocations can provide insight into the methods used to establish populations, assess the diversity in translocations against that of natural subpopulations and, if required, provide guidance for augmentations aimed to boost the genetic diversity within subpopulations (Van Rossum & Hardy 2022).

One of many threatened species in Victoria with a restricted range is *Grevillea celata* Molyneux (Nowa Nowa/Colquhoun grevillea) which is limited to an area of only 60 km$^2$ in eastern Victoria and listed as Critically Endangered under the Victorian Government *Flora and Fauna Guarantee Act 1988* (FFG) (DEWLP, 2021). A translocation was undertaken prior to 2009 in response to roadworks (DSE, 2009) but only 3–5% of translocated plants remain. This research examines the genetic diversity across the range of *G. celata* and estimates the level of connectivity amongst remaining natural subpopulations. This information can be used to prioritise management actions that preserve genetic diversity *in situ* and guide *ex situ* collections to maximise genetic diversity conserved. A complete picture of the genetic diversity in *G. celata* can also guide genetic rescue of small, isolated subpopulations by ensuring that the source for augmentation is the most appropriate to maintain any unique diversity or potential local adaptation that may be present within subpopulations.

## Methods

### Species

*Grevillea celata* (Nowa Nowa/Colquhoun grevillea) is endemic to Victoria where it is restricted to well-drained sandy soils in the Colquhoun State Forest between Nowa Nowa and Bruthen in central east Gippsland. *Grevillea celata* is an erect and open, low and dense, root-suckering shrub 0.4–1.8 m high (Makinson, 1996). Red and yellow flowers up to 12 mm long appear from July to February (Figure 1). The species is most likely pollinated by birds and insects with ants observed collecting and burying seed, potentially an important component for germination (Molyneux, 1995). It is very similar to two other more widespread grevilleas, *G. chrysophaea* and *G. alpina*. *Grevillea chrysophaea* (FFG Vulnerable) has yellow flowers and does not root-sucker. It grows in eucalypt woodland or heath on silty sand to sandy loam with a disjunct distribution in the Brisbane Ranges (Anakie-Steiglitz area) and Gippsland, in the area roughly enclosed by Traralgon, Woodside and Sperm Whale Head-Licola. *Grevillea alpina* is widespread in Victoria and on the southern Tablelands of New South Wales (Australia's Virtual Herbarium, 2022). Preliminary phylogenetic studies place *G. celata* nested within Gippsland collections of *G. chrysophaea*, however, the relationship between the two has not been fully resolved (Holmes 2021).

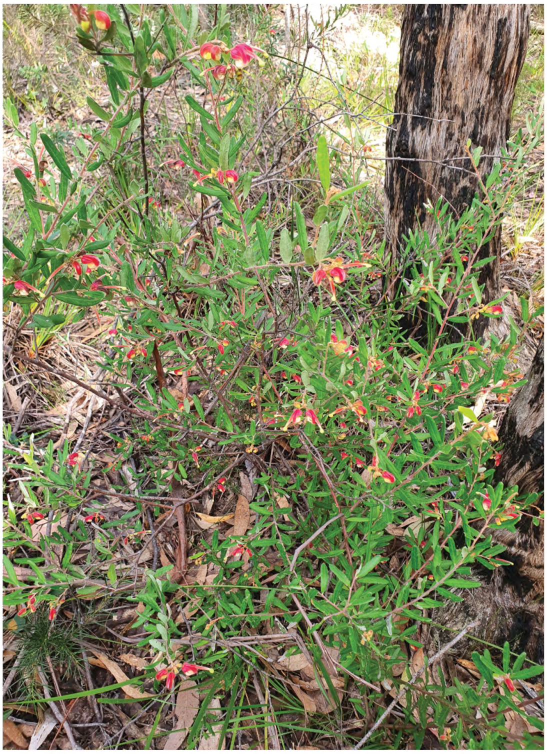


**Figure 1** *Grevillea celata.*

Surveys of *G. celata* in 2006 estimated 1,500 plants occurring in nine wild populations across a range of 11 km (60 km$^2$) (Carter & Walsh, 2006), with no populations occurring in conservation reserves. Due to extreme range restriction and small population size, *G. celata* has been listed as critically endangered under the *Victorian Flora and Fauna Guarantee Act* (Vic) (DELWP, 2021). This species was assigned a genetic risk rating of high in the 2020 report assessing genetic risk to Victorian flora and fauna (Kriesner *et al*. 2020).

### Sampling for population genetic analyses

Leaf samples were collected from *G. celata* plants targeting the nine discrete locations described in the National Reco55very Plan (Carter & Walsh 2006) and an additional five locations based on National Herbarium of Victoria records or Atlas of Living Australia observation records (Australia's Virtual Herbarium, 2022) (Figure 2). The Action Statement for *G. celata* described the destruction of a population while upgrading the Bruthen-Nowa Nowa Road (DSE 2009). To offset this loss, a translocation of 1000 plants was undertaken at a nearby location with the area fenced off for protection. The assumed location was scouted during field surveys, with approximately 30–50 plants observed in August 2021 within a damaged fence. Translocated plants were purposefully not sampled for this genetic study to reduce the chance of bias of including clonal (cutting-propagated plants) samples in the data. The widespread bushfires in Australia in 2019/2020 affected the following five collection locations in Colquhoun State Forest north of Bruthen-Nowa Nowa Rd: Watershed Rd, sites 1 and 2 (WATR1/WATR2), Unnamed track, North and South side

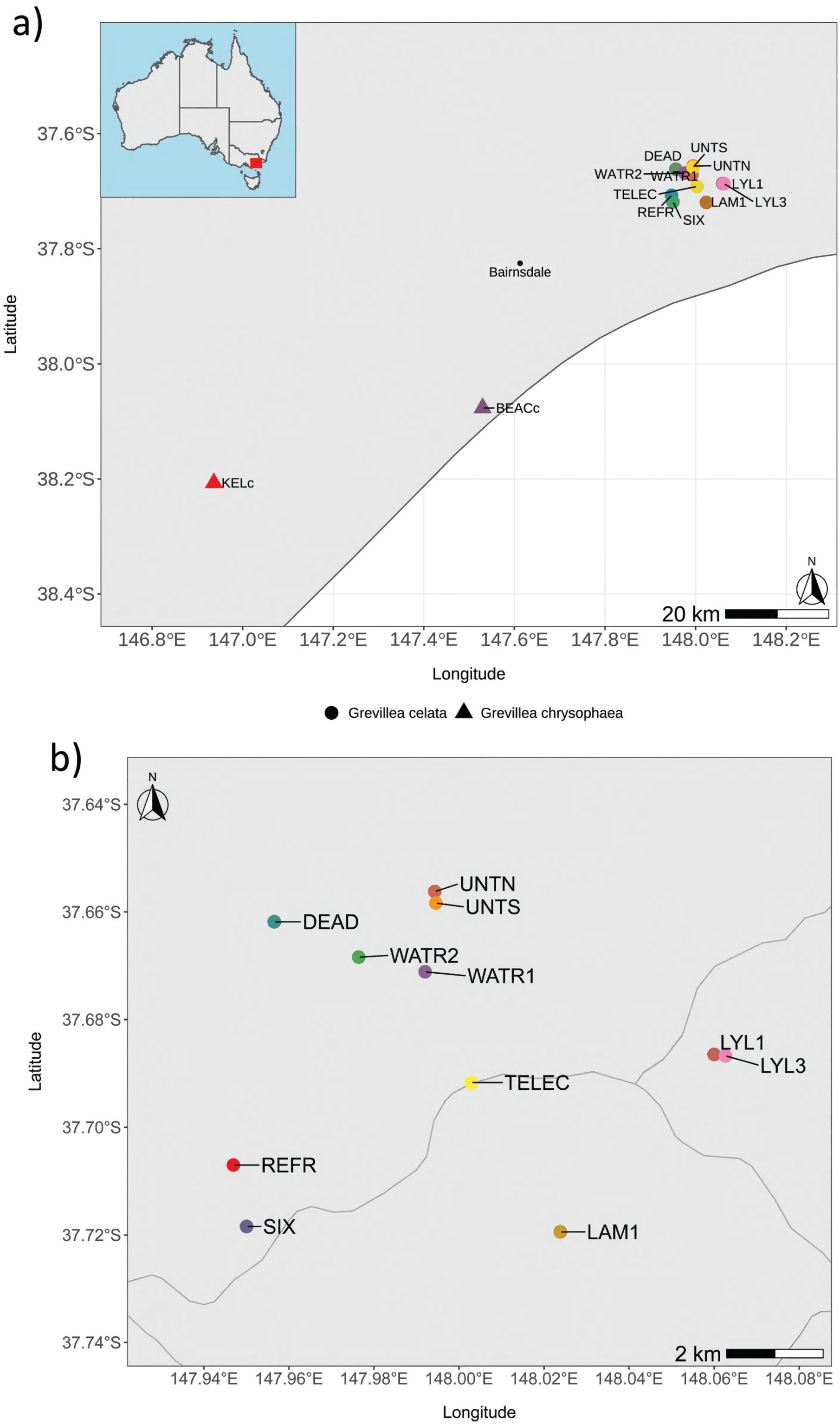

a)
37.6°S
37.8°S
38.0°S
38.2°S
38.4°S
Latitude
UNTS
DEAD
UNTN
WATR2
WATR1
LYL1
TELEC
LAM1
LYL3
REFR
SIX
Bairnsdale
BEACc
KELc
N
20 km
146.8°E
147.0°E
147.2°E
147.4°E
147.6°E
147.8°E
148.0°E
148.2°E
Longitude
● Grevillea celata ▲ Grevillea chrysophaea
b)
N
37.64°S
37.66°S
37.68°S
37.70°S
37.72°S
37.74°S
Latitude
UNTN
UNTS
DEAD
WATR2
WATR1
LYL1
LYL3
TELEC
REFR
SIX
LAM1
2 km
147.94°E
147.96°E
147.98°E
148.00°E
148.02°E
148.04°E
148.06°E
148.08°E
Longitude

**Figure 2** Map of study area and locations of sampled populations, a) *Grevillea chrysophaea* populations in relation to *Grevillea celata* study area, b) sampled *Grevillea celata* populations across its distribution.

of Stony Creek Crossing (UNTN/UNTS) and Dead Horse Creek Rd (DEAD). In these locations, plants appeared to be resprouting and suckering from root stock, with regrowth to 1 m tall and flowering observed at all locations. In many unburned locations, plants were also observed to be suckering, with large plants surrounded by small plants assumed to be ramets within a 2 m area. More recent surveys confirmed that plants were setting fruit, indicating that the species is not reliant on asexual reproduction for persistence; seed collections have been banked at the Victorian Conservation Seedbank (MEL 2510915, 2372894).

Clonal reproduction via root-suckering was confirmed by genetic analysis of 4 ramets sampled less than 2 metres apart. These samples were later removed from further analyses. In all other locations, sampling was undertaken of plants believed to be separate individuals spaced at least 10 m apart across the geographic extent of a site. At two small and isolated sites, the Corner of Lambournes and Nowa Nowa-Bruthen Road (LAMBRU, 20 stems), and the edge of Nowa Nowa-Bruthen Road (8MIL, 6 stems), it was necessary to sample at intervals of less than 10 m. At Six Mile Track (SIX), plants occurred at a lower density and were spread across a greater distance than at other locations. It was necessary to spread individual collections across 1.35 km to sample five individuals that appeared to be separate plants.

During field collections, it was noted that in some large populations, plants formed discrete patches separated by 100–200 m. Due to this intermittent distribution, two patches were sampled at two locations to quantify the differentiation between patches within locations. At both Lyles Break/Smarts Break intersection (LYL1/LYL3) and Unnamed track, N and S side of Stony Creek Crossing (UNTN/UNTS), we collected from two discrete patches located approximately 250 m apart at LYL1/LYL3, and approximately 100 m at UNTN/UNTS. In addition, two of the geographically closest populations of *G. chrysophaea*, Holey Plains State Park (KELc) and Gippsland Lakes Coastal Park (BEACc) were sampled to test whether populations of *G. celata* were distinct entities from *G. chrysophaea* (Figure 2a).

## DNA extraction, amplification, and sequencing

Silica-dried leaf samples (94 samples including technical repeats) were sent to a commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia), for DNA extraction and DArTSeq analysis, a reduced representation sequencing method (Kilian *et al*. 2012). DNA was extracted at DArT using the Nucleo Mag kit (Machery Nagel, Germany), on a Tecan 100 platform following the manufacturers protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and MseI and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina NovaSeq X+ sequencer. Sequences were processed using proprietary DArT analytical pipelines to filter poor quality sequences. Resultant sequences were used in DArTsoft14, DArT PL's proprietary SNP calling algorithms.

## Data analysis

The unfiltered SNP dataset received from DArT consisted of 101665 SNPs and 94 samples. To identify clonal samples, the dataset was assessed for (1) genetic distance, using the R package 'stats' to calculate pairwise Euclidean distances between samples, (2) kinship, using KING method of moments using the R package 'SNPRelate', and (3) proportion of shared alleles, using the R package dartR. For the genetic distance and proportion of shared alleles methods, the included repeats (known clonal samples) were used to set a threshold to select clonal samples. The threshold was calculated as the average value between repeat pairs plus/minus three standard deviations of the repeat pair values. Any sample pairs below these values in the genetic distance measure or above in the proportion of shared alleles were considered clonal samples for that method. KING method of moments identified monoclonal individuals. If pairs of samples were identified as clonal in at least two of these methods, they were considered clonal. Monoclonal populations (LAMBRU and 8MIL), and one individual from each pair of samples identified as ramets, and the samples used to test for assumed clonal growth (LAM2) were removed from further analysis, see results.

Two datasets were designated and analysed: the first dataset contained all remaining *G. celata* populations and the two *G. chrysophaea* populations; the second dataset contained only *G. celata* populations. The resultant datasets were filtered using the R package dartR (Gruber *et al*. 2018) in R (R Core Team, 2019). Data were filtered to a locus call rate of 80% and individual call rate of 75%, a reproducibility score of 1, a Hardy-Weinberg equilibrium with a 5% level of significance, a minor allele frequency greater than 4%, secondaries were removed as were monomorphic loci and then filtered on Hamming distance to remove potential paralogues.

For the first dataset, a principal component analysis (PCA) was undertaken on all *G. celata* and *G. chrysophaea* samples (clones removed) to determine whether *G. celata* populations are genetically distinct entities from *G. chrysophaea*. The following analyses were undertaken on the second dataset. To identify genetic clusters of individuals and visualise the major axes of variation between clusters, PCA was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011) in R (R Core Team, 2019). Expected and observed heterozygosity, private alleles, inbreeding coefficients and pairwise population differentiation ($F_{ST}$) were assessed using the adegenet (Jombart & Ahmed 2011), hierfstat (Goudet & Jombart, 2020) and Poppr (Kamvar *et al*. 2014, 2015) packages in R. Analysis of molecular variance (AMOVA) was tested using the Poppr (Kamvar *et al*. 2014, 2015) package in R. Isolation by distance (IBD) was assessed using R package dartR (Gruber *et al*. 2018). Population genetic structure was explored using Structure 2.3.4 (Pritchard *et al*. 2000). Ten independent runs were undertaken for each K value from 1 to 13 with a burn-in of 200,000 and 300,000 MCMC iterations. The R package pophelper (Francis 2017) was used to visualise results and select the most probable K based on the ΔK metric (Evanno *et al*. 2005). The R package ggplot2 (Wickham 2016) was used to visualise results. The R package diverRsity (Sundqvist *et al.* 2016) was used to estimate migration rates, with plotting using igraph (Csardi & Nepusz 2006) and qgraph (Epskamp *et al*. 2012).

# Results

## Clonal/suckering growth

The test for clonality at site LAM2 demonstrated that all four stems presumed to be sucker growth were found to be ramets from the same genetic individual, confirming that suckering is a strategy for this species. The four samples had an average pairwise genetic distance of 80.6 and an average proportion of shared alleles value of 0.97, while the average values between the unrelated individual and these samples were 217.6 and 0.85 respectively.

Clonal analysis across all samples identified two sites as monoclonal, LAMBRU and 8MIL, with average pairwise genetic distance values within the site of 60.6 and 89.4 and average proportion of shared alleles value of 0.98 and 0.97 for LAMBRU and 8MIL respectively. In contrast, the average pairwise genetic distance amongst all unrelated samples was 236 and average proportion of shared alleles values was 0.83. Two other pairs of samples in two separate subpopulations (SIX, LAM1) were also identified as clonal, with pairwise genetic distance values of 71.3 and 99.6 and proportion of shared alleles value of 0.98 and 0.96.

## Species delimitation

The results from DArTSeq contained 63 samples and 97013 loci. After filtering, as outlined above, the SNP data set contained 10129 loci and 59 individuals across 13 locations. The PCA of the *G. celata*/*G. chrysophaea* dataset confirms that the *G. celata* samples are genetically distinct from the two sampled locations of *G. chrysophaea* (Figure 3). The first axis of the principal component analysis differentiates the two species, explaining 17.16% of the variation in the dataset. The second axis explains 4.68% of the differentiation and separates the discrete locations of *G. chrysophaea* sampled.

## Genetic diversity of *Grevillea celata*

The results from DArTSeq contained 53 samples and 73743 loci. After filtering, as outlined above, the SNP data set contained 9290 loci and 50 individuals across 11 locations. Spread of final locations in the analysis are shown in Figure 2b. Analysis of *G. celata* found overall nuclear genetic diversity to be 0.236. Gene diversity values at individual locations are shown in Table 1. Expected heterozygosity levels were consistent across all locations, except for LAM1 which was lower. Observed heterozygosity was more variable (ranging from 0.106 to 0.162 +/- 0.002 SE) and lower in every site than expected heterozygosity (ranging from 0.155 to 0.192 +/- 0.002 SE). This is reflected in the inbreeding coefficient

**Table 1 Genetic diversity characteristics of the sites including number of sampled individuals in final dataset, number of private alleles, expected heterozygosity, observed heterozygosity and inbreeding coefficient for each of the populations, standard error in brackets.**

| | Number of individuals | No. private alleles | Expected heterozygosity | Observed heterozygosity | Inbreeding coefficient |
|---|---|---|---|---|---|
| REFR | 5 | 15 | 0.180 (0.002) | 0.125 (0.002) | 0.305 |
| SIX | 4 | 44 | 0.176 (0.002) | 0.162 (0.002) | 0.079 |
| DEAD | 5 | 19 | 0.187 (0.002) | 0.133 (0.002) | 0.290 |
| WATR2 | 5 | 24 | 0.189 (0.002) | 0.161 (0.002) | 0.147 |
| WATR1 | 5 | 24 | 0.184 (0.002) | 0.146 (0.002) | 0.208 |
| UNTN | 5 | 10 | 0.192 (0.002) | 0.157 (0.002) | 0.178 |
| UNTS | 4 | 6 | 0.182 (0.002) | 0.154 (0.002) | 0.154 |
| TELEC | 5 | 11 | 0.187 (0.002) | 0.143 (0.002) | 0.232 |
| LAM1 | 4 | 7 | 0.155 (0.002) | 0.106 (0.002) | 0.313 |
| LYL1 | 4 | 9 | 0.175 (0.002) | 0.153 (0.002) | 0.128 |
| LYL3 | 4 | 12 | 0.175 (0.002) | 0.150 (0.002) | 0.142 |

(ranging from 0.079 to 0.313), with LAM1, DEAD and REFR showing the highest levels of inbreeding. Site SIX showed the lowest inbreeding value while also having the highest number of private alleles.

## Genetic structure and differentiation

The first two principal components explained 8.9% of variance in the genotypic data (Figure 4). The first axis of the PCA (4.79%) identifies the variation amongst individuals at all locations, which cluster according to the sampling location, spread across the first axis resembling the spatial spread of the locations from East to West. The second axis indicates there is a large amount of variation within population SIX which accounts for the differentiation seen in axis 2 (4.11%). The variation within SIX is greater than that which separates UNTN/UNTS from locations to its south (WATR1/2). The paired locations (LYL1/LYL3 and UNTN/UNTS) cluster together as expected based on their geographic proximity, as do samples from WATR1, WATR2 and DEAD.

Population genetic structure, assessed in Structure,

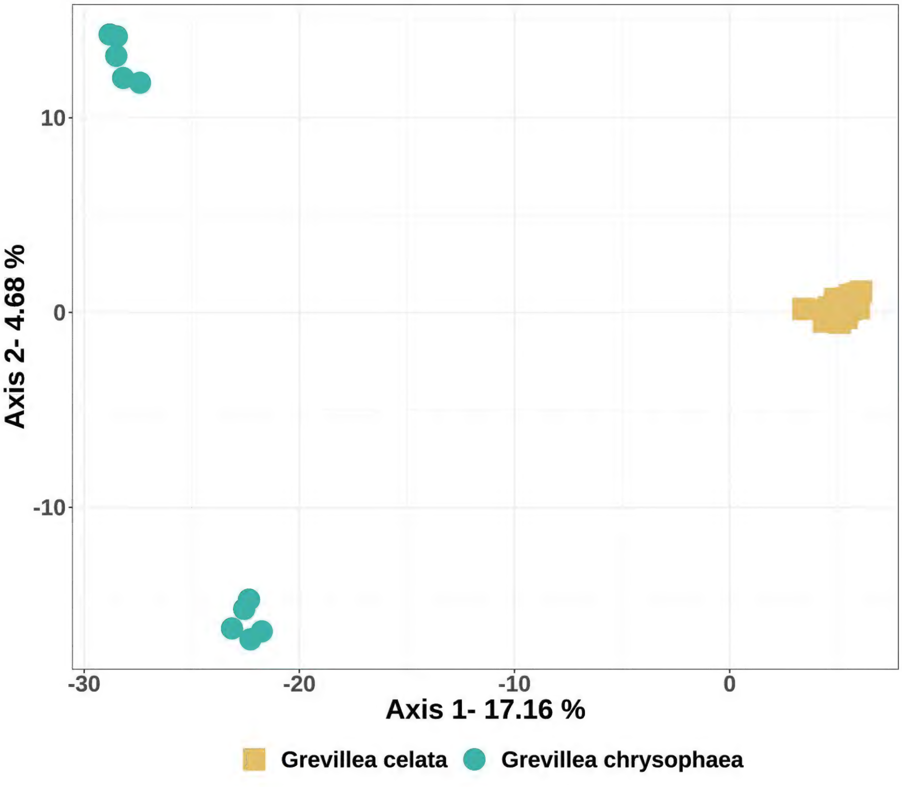


**Figure 3** Plot of first and second component of principal component analysis of genetic differentiation for species delimitation between *Grevillea chrysophaea* and *Grevillea celata*.

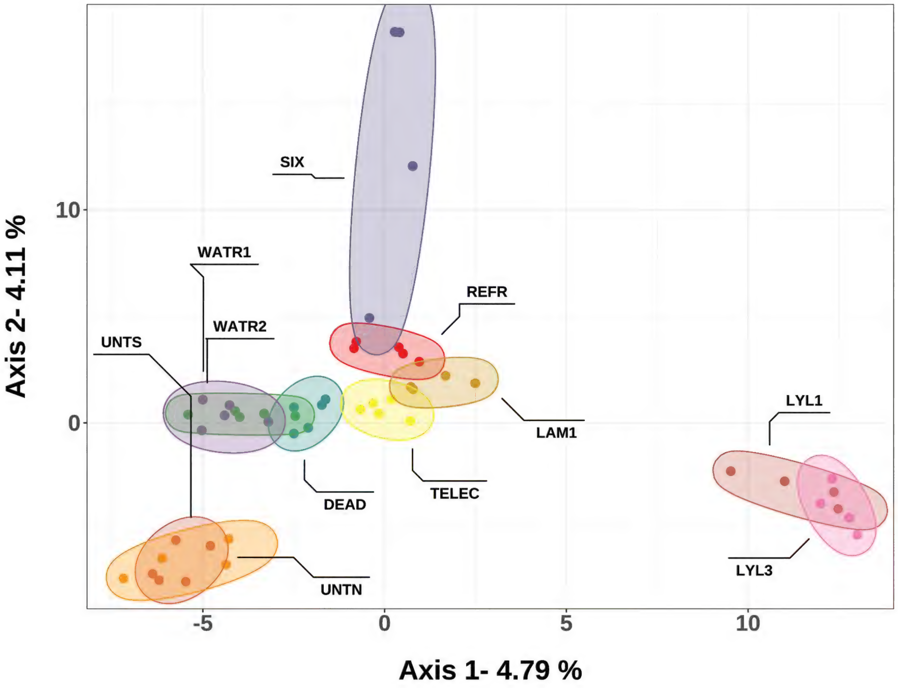


**Figure 4** Plot of first and second axis of results from principal component analysis of genetic differentiation for *Grevillea celata*.

identified four genetic clusters with similar genetic patterns to the PCA results (Figure 5). The paired locations UNTN/UNTS and LYL1/LYL3 each form a cluster and most individuals from SIX form a distinct genetic cluster. The remaining locations are primarily composed of the same genetic cluster, with admixture in many individuals across these locations. Individuals at DEAD and WATR1/2 show admixture primarily with the UNTN/UNTS genetic group reflecting their close geographic proximity. Individuals at TELEC and LAM1 show admixture largely with the LYL genetic group. REFR shows admixture with the LYL group to a lesser extent, indicating a reduced gene flow at a greater distance, supporting the isolation by distance results.

Overall species genetic differentiation ($F_{ST}$) was found to be 0.0865. Measures of pairwise $F_{ST}$ ranged from a low of 0.004 to a high of 0.16 (Figure 6). The highest differentiation values were between SIX and LYL, locations 10 km apart at opposite longitudinal ends of the species distribution. SIX also had higher $F_{ST}$ values with locations half that distance apart (6-7.9 km, UNTN/UNTS and WATR1/WATR2. The lowest differentiation was between the paired locations UNTN and UNTS (0.004) which were the closest in geographic distance. The paired populations LYL1 and LYL3 also had a low differentiation (0.046), but this was greater than that between TELEC and REFR (0.035) which are located a greater distance apart. This is supported by the isolation

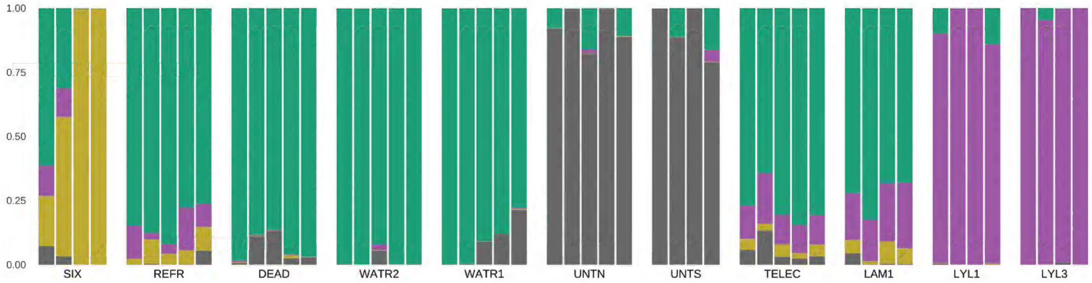
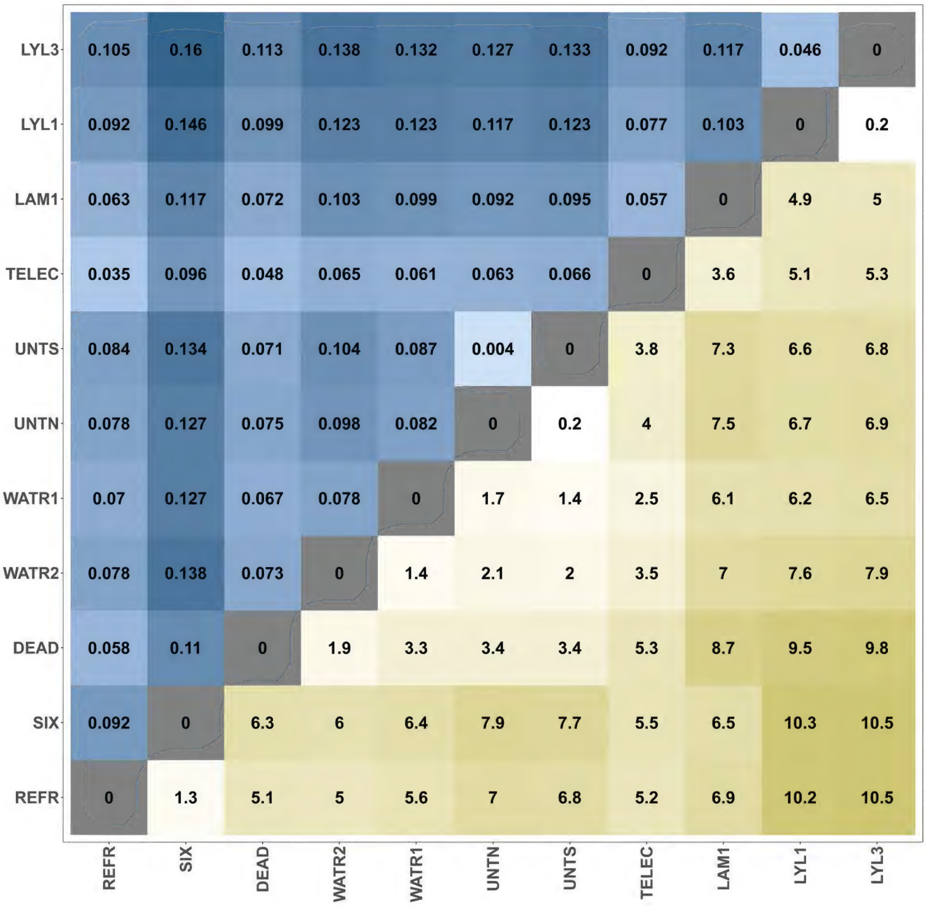


**Figure 5** Genetic clustering results from Structure analysis for *Grevillea celata* sites. Each individual is represented by a vertical bar which is apportioned into its membership to each of the identified four genetic clusters.



| | REFR | SIX | DEAD | WATR2 | WATR1 | UNTN | UNTS | TELEC | LAM1 | LYL1 | LYL3 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LYL3 | 0.105 | 0.16 | 0.113 | 0.138 | 0.132 | 0.127 | 0.133 | 0.092 | 0.117 | 0.046 | 0 |
| LYL1 | 0.092 | 0.146 | 0.099 | 0.123 | 0.123 | 0.117 | 0.123 | 0.077 | 0.103 | 0 | 0.2 |
| LAM1 | 0.063 | 0.117 | 0.072 | 0.103 | 0.099 | 0.092 | 0.095 | 0.057 | 0 | 4.9 | 5 |
| TELEC | 0.035 | 0.096 | 0.048 | 0.065 | 0.061 | 0.063 | 0.066 | 0 | 3.6 | 5.1 | 5.3 |
| UNTS | 0.084 | 0.134 | 0.071 | 0.104 | 0.087 | 0.004 | 0 | 3.8 | 7.3 | 6.6 | 6.8 |
| UNTN | 0.078 | 0.127 | 0.075 | 0.098 | 0.082 | 0 | 0.2 | 4 | 7.5 | 6.7 | 6.9 |
| WATR1 | 0.07 | 0.127 | 0.067 | 0.078 | 0 | 1.7 | 1.4 | 2.5 | 6.1 | 6.2 | 6.5 |
| WATR2 | 0.078 | 0.138 | 0.073 | 0 | 1.4 | 2.1 | 2 | 3.5 | 7 | 7.6 | 7.9 |
| DEAD | 0.058 | 0.11 | 0 | 1.9 | 3.3 | 3.4 | 3.4 | 5.3 | 8.7 | 9.5 | 9.8 |
| SIX | 0.092 | 0 | 6.3 | 6 | 6.4 | 7.9 | 7.7 | 5.5 | 6.5 | 10.3 | 10.5 |
| REFR | 0 | 1.3 | 5.1 | 5 | 5.6 | 7 | 6.8 | 5.2 | 6.9 | 10.2 | 10.5 |

**Figure 6** Pairwise genetic distance ($F_{ST}$) among populations of *Grevillea celata* coloured in blue in the upper triangle and geographic distance (km) among populations coloured in khaki in the lower triangle. Higher values (greater differentiation/ distance) are shaded darker.

by distance (IBD) results which found a highly significant relationship between genetic and geographic distance (Mantel statistic = 0.587, $p = 0.001$), suggesting that gene flow diminishes with distance. Analysis of molecular variance (AMOVA) showed that the majority of genetic variation was found within samples with 61.76% ($p$ = 0.001) of the variation explained whereas only 9% ($p$ = 0.001) was explained between populations.

## Discussion

*Grevillea celata* has a restricted range and is represented by a small number of subpopulations. Whilst the species delimitation undertaken in this research was not a formal phylogenetic analysis, the result indicates that the *G. celata* subpopulations are genetically distinct from the nearest *G. chrysophaea* subpopulations and thus represent a conservation unit deserving of management. Asexual reproduction, through root-suckering and resprouting, was confirmed in the species, so population census counts may not reflect the effective population size. This root-suckering trait is useful after fires enabling standing numbers to rebound quickly, avoiding the loss of genetic diversity and a reliance on a seedbank. However, sexual reproduction is important as it allows for genetic recombination needed for evolution and adaptation to changing environments (Bürger 1999).

Genetic analysis has revealed the relationships both within sites and across the overall population and the importance of understanding reproductive strategies prior to the development of *ex situ* living collections or seedbanks for restoration activities. Genetic assessment of *G. celata* has highlighted locations that are genetically depauperate and could benefit from genetic rescue practices which should improve plant fitness and resilience (Frankham 2015) while also improving the evolutionary potential by reducing loss of genetic diversity from small populations through drift.

In a review of Australian threatened flora, 40% of 1,135 threatened taxa were assessed as actively declining and of the highest conservation concern (Silcock & Fensham 2018). A further 40% were narrow-range endemics that meet the IUCN assessment criteria for EN (Endangered) or CR (Critically Endangered) but are not considered to be actively declining (Silcock & Fensham 2018). *Grevillea celata* is a naturally range-restricted species and while numbers in remnant populations may not be actively declining, such narrow-range species are at a high risk of being affected by a single catastrophic event such as a fire that could affect the entire range. The main fire response strategy of *G. celata* is to resprout or root-sucker, which was observed in locations north of Bruthen-Nowa Nowa Rd that were severely impacted by fire. Whilst this indicates successful post-fire recovery, if a large fire occurred across the entire species' range or repeated fires occurred at the same locations, such events could result in an overall decline of the species.

Globally, fire activity is projected to increase, and fires are predicted to become larger and more severe in south-eastern Australia (Collins *et al*. 2022; Ellis *et al*. 2022). A species' reproductive success is critical to landscape persistence and is influenced by fire regimes, seedbanks, and disturbance. Managers require a better understanding of those interactions to guide conservation of endangered species particularly in the face of global environmental changes (Silcock & Fensham 2018). The recovery plan for the species (Carter & Walsh 2006) suggests that a 10-year fire cycle may be appropriate for *G. celata* as this would allow time for plants to mature and seed to accumulate in the soil and would reduce competition with fire-promoted species such as Bracken (*Pteridium esculentum*). More frequent fires may promote suckering of plants rather than recruitment via seed as demonstrated in *G. rhizomatosa* (Gross & Caddy 2006). The relative contribution of sexual and asexual reproduction in *G. celata* is not known, however viable seed is produced. Although *G. celata* is capable of resprouting after fire, it is expected that long term population persistence requires some sexual reproduction to occur between fires.

All sampled sites of *G. celata* show some level of inbreeding, indicating that gene flow is restricted across the range, confirmed by the positive correlation between genetic and geographic distance. Smaller occurrences or clusters of plants in the west of the range (SIX, 8MIL) may be less attractive to birds as pollinators, suggesting pollination may be primarily mediated by insects that travel shorter distances. The collection and dispersal of seed by ants limits long distance dispersal, however pollination by insects and birds should lead to longer distance pollen-mediated gene flow (Molyneux 1995). While more isolated, LYL1 and LYL3 in the east of the range is large with denser occurrences of stems and therefore should be capable of attracting pollinators and temper inbreeding within the site. The isolation of this site may be preventing the longer-range movement between populations. While it appears that migration levels amongst *G. celata* are great enough to prevent the formation of high levels of genetic differentiation, distinct genetic clusters are present, likely driven by localised breeding and adaptation in response to local conditions. It is important to preserve the natural genetic clustering within the species, whilst undertaking management actions such as augmentation to ensure local adaptation is not lost through genetic swamping.

Our results showed that some *G. celata* locations with a small number of stems (LAMBRU, 8MIL) can represent either a single or very few genetic individuals. While plants were flowering at these locations, they may be persisting primarily through asexual reproduction. Unrecognised clonality through root-suckering means that census counts could over-estimate the number of genetic individuals present at any given location of *G. celata*. Plants at SIX harbour unique genetic diversity and greater variation within this location despite occurring at a low density across a large distance. Here, the plants do not seem to have spread asexually and only a few stems/plants are present, potentially a reflection of a lack of disturbance or fire in this part of the species range. A subpopulation with so few individuals is prone to inbreeding effects and the associated long term risks affecting persistence (Ellstrand & Elam 1993). The group of individuals from SIX on the edge of the species range is not geographically isolated as another sampled population occurs just over a kilometre away and yet it has three individuals with a unique genetic signature. It is important to assist the small population of *G. celata* (SIX) to become sustainable by increasing the number of individuals through genetic rescue, whilst ensuring that any augmentation does not swamp the unique diversity represented by just three individuals (Bragg *et al*. 2021; Frankham 2015; Whiteley *et al*. 2015). In such a scenario, plants could be sourced from REFR, which has historical admixture with the unique genetic cluster detected at SIX.

Genetic monitoring is an important tool for assessing translocation success, as sexual or asexual recruitment methods can be determined (Van Rossum & Hardy 2022). Historic translocations of *G. celata* were undertaken with the best intention of establishing plants at a new location to replace those lost due to roadworks. Through discussions with botanists at Royal Botanic Gardens Victoria (RBGV) about interpreting the *G. celata* results, it was revealed that the genetically identical site LAMBRU, is the surviving remains of a translocation to compensate for plants destroyed by road upgrades (DSE 2009). The genetic uniformity of the samples collected from this location indicates that the translocated plants were propagated from cuttings of one individual and do not reflect a genetically healthy population. Rapid genetic decline of translocated populations is likely if the founder individuals are from a limited genetic background (Krauss *et al*. 2002). It is therefore recommended for any species that any translocations incorporate a genetically diverse germplasm to alleviate the chance of inbreeding (Commander *et al*. 2018). The LAMBRU site is an ideal candidate for augmentation to 'genetically rescue' this established subpopulation (Whiteley *et al*. 2015). The single genotype at LAMBRU clusters with the central genetic group (data not shown) making DEAD, WATR, REFR ideal source locations. This finding demonstrates the direct application of genetic analysis in assisting appropriate conservation actions, as the clonal nature of LAMBRU would not have been known without genetic assessment as it otherwise looks to be a healthy population when assessed by population census alone.

## Implications for conservation

The establishment of an *ex situ* collection of at least 30 mature plants in cultivation is detailed in the recovery actions for *Grevillea celata* (Carter & Walsh 2006). While cutting propagation techniques are a successful way to produce large numbers of plants in a short period of time, those plants are genetic clones of their source plant. Therefore, efforts must be made to collect from multiple plants to maximise sampling unique individuals to capture sufficient diversity from a site and to reduce the chance of over-representation of one clone in the *ex situ* collection (Commander *et al*. 2018; Guerrant *et al*. 2014). *Ex situ* collections of *G. celata* should encompass germplasm collected from multiple plants within the four geographic clusters described above (LYL, LAM, SIX and DEAD/WATR/REFR) to maximise the *ex situ* representation of genetic diversity. Seed collections should be made from as many plants as possible within each geographic cluster with seed lots from locations stored separately, ideally as maternal lines, to maintain control over genotype selection (Commander *et al*. 2018). The populations identified with low, or no genetic diversity will be best managed by augmentations with genetically diverse plants. Due to the genetic structuring, it would be advisable that any augmentation is done with plants from the same genetic cluster, unless this is not a viable option (SIX). Utilising the genetic findings from this study in future management activities should maximise the genetic diversity in populations resulting in healthier populations best prepared for future challenges.

Further phylogenetic research is underway on the *Grevillea Arenaria* and *Floribunda* subgroups and future work may resolve *G. celata* at a different taxonomic rank (Holmes 2021). However, the work undertaken here indicates that *G. celata* as currently recognised constitutes a discrete conservation unit and thus *in-* and *ex situ* management actions are warranted to preserve the unique diversity. Conservation of this range-restricted species needs to incorporate the above recommendations derived from the results of this genetic analysis. *In situ* management, such as the augmentations detailed above, will strengthen genetic diversity across the range, ensuring the greatest potential for future survival. These results have already guided the establishment of *ex situ* conservation collections at RBGV aimed at preserving maximum genetic diversity of the species. A living collection representative of the four major genetic clusters is currently being cultivated at RBGV, with material taken from multiple plants spread well across locations to avoid the collection of root-suckering individuals. Currently seed is held in the Victorian Conservation Seedbank from two of the four genetic clusters, with future additional seed collections guided by the above results to support the long term *ex situ* conservation of *G. celata*. These *ex situ* collections provide insurance for this Critically Endangered, range-restricted species in the case of disastrous loss of individuals through range wide catastrophic events.

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# High levels of clonality in *Pomaderris oblongifolia* and *Pomaderris buchanensis* (Rhamnaceae): implications for conservation

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## Abstract

*Pomaderris oblongifolia* N.G.Walsh and *P. buchanensis* N.G.Walsh are two rare, range-restricted species from Victoria's Snowy River region composed of small, fragmented subpopulations. To assist conservation management, genetic variation was quantified and identified high levels of clonality in both species, as well as in the related, sometimes sympatric, species *P. aspera* Sieber ex DC. Genetic data for *P. buchanensis* were consistent with triploidy, a common trait in the genus. The close relationship between sympatric *P. buchanensis* and *P. aspera*, revealed by kinship analysis suggests that *P. buchanensis* may be a triploid variant derived directly from *P. aspera*. Studies to determine the breeding system, apomixis and ploidy levels would be beneficial for guiding the assembly of genetically representative *ex situ* germplasm collections.

**Keywords:** population fragmentation, rare species, SNPs

## Introduction

Conservation of highly fragmented range-restricted flora species is challenging as they are susceptible to genetic decline in addition to having a higher risk of extinction compared to more widespread species with continuous distributions (Ellstrand and Elam 1993; Broadhurst *et al*. 2017; IUCN SPC 2024). Two of the main components of the Common Assessment Method (CAM) are species' range size and population size. CAM is used in Australia to evaluate a species' risk of extinction against the International Union for Conservation of Nature (IUCN) Red List Categories (TSSC 2021; IUCN SPC 2024). Geographic range size is a strong predictor of a species' extinction risk because the impact of local loss of individuals and habitat is minimised if a species range is large, and the chance of range-wide impacts is reduced (Hamrick and Godt 1989; Gaston & Fuller 2009; LeBreton *et al.* 2019). A lack of genetic connectivity between disjunct populations can increase population genetic differentiation, which may increase the chance of local adaptation and reduce the adaptive ability of populations (Aitken & Whitlock 2013; Broadhurst *et al*. 2017; Hopley & Byrne 2019; Pennington *et al*. 2021).

The second component, estimating population size based on a census of the number of mature (adult) individuals, is integral to the

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CAM, as breeding population size determines the ongoing regeneration of a species, the maintenance of genetic diversity across generations and the effects of demographic stochasticity (Lande 1993; Frankham 2005; TSSC 2021; IUCN SPC 2024). However, the use of population census data generated by direct observation for conservation assessments and activities can be problematic without considering the effects that reproductive biology may have on the genetic diversity of a species. Plants commonly violate the theoretical expectations of genetic diversity through mixed mating systems (selfing, outcrossing), asexual reproduction and polyploidy (Kireta *et al.* 2019; Rice *et al*. 2019).

Polyploidy can bestow potential survival advantages through heterosis, greater adaptability via a higher number of alleles per locus and the production of asexual seed by apomixis. However, they also need to persist through genomic instability and not be sterile triploids or higher oddploids (reviewed in Comai 2005). Ancient polyploidy, followed by subsequent re-diploidisation are responsible for the radiation and diversification of angiosperm flora (Comai 2005; Rice *et al*. 2019; Nge et al. 2024). Polyploid plants may have traits that provide a higher competitive advantage compared to diploid species (Chan *et al.* 2022). Asexual reproduction, such as root-suckering, is implicated in the persistence of marginal, rare and/or endangered species and as a mechanism to cope with sexual reproductive failure but does not promote dispersal (Vallejo-Marín *et al*., 2010; Bradbury *et al*., 2015; Millar and Byrne 2020). Apomixis may compensate for sexual failure, enabling dispersal via established seed dispersal pathways and is often associated with polyploids and implicated in the evolutionary success of species in complex environments (Chen *et al*. 2019; Delnevo *et al.* 2023). Even though asexual reproduction and polyploidy may enable rapid population growth, offer reproductive insurance and better colonising abilities, both mechanisms influence fecundity and genetic recombination, which can lead to populations comprising only a small number of genetically unique individuals (Warburton *et al*. 2000; Hojsgaard and Hörandl, 2019; Delnevo *et al*. 2023; Simmons *et al*. 2023). Thus, if asexual reproduction is present, deriving effective population size by direct observation alone may overestimate the number of genetically unique individuals present in a population (Warburton *et al*. 2000; Simmons *et al*. 2023) and may underestimate the levels of genetic diversity, inbreeding and declining evolutionary potential in a species (Fisher 1930; Wright 1931; Young *et al*. 2002; James and McDougall 2014). Conservation *in situ* and *ex situ* therefore relies upon knowledge of levels of clonality, true breeding population size and the relatedness between individuals (Hoban 2019; Hoban and Strand 2015).

*Ex situ* conservation collections such as long-term storage of seeds play a strong role in the conservation of a given species and its genetic diversity (IUCN/SSC 2014). The function of many *ex situ* collections is to act as a genetically diverse insurance collection that can be used as a source of germplasm for population restoration (Martyn Yenson *et al*. 2021). In the absence of genetic information, generalised advice proposes that germplasm collections are made from at least 50 individuals across sites (or maximum number of individuals if less than 50; Martyn Yenson *et al.* 2021), as minimum viable population size is often estimated at 50 individuals (Jamieson and Allendorf 2012). Ideally, the diversity in *ex situ* collections should be comparable to that *in situ* as it provides the basis for a species' restoration. Uneven population sampling, low genetic diversity in cultivation, and ongoing cultivation can affect the genetic integrity of *ex situ* germplasm (Diaz-Martin *et al.* 2023) and lead to reduced fitness in cultivation (Forgiarini *et al.* 2023). While asexual reproduction via rhizomes or root-suckering may be visible, clonality via apomixis can be cryptic in the absence of genetic data as plants produce viable seed but the collection of seed from multiple plants does not ensure genetically variable collections as seeds maintain the parental genotype (Koltunow and Grossniklaus 2003; Martyn Yenson *et al*. 2021). Species-specific outcomes will be improved if germplasm collections can be designed around a taxon's landscape genetic patterns and fitness (Chen *et al.* 2019; Garner *et al*. 2020; Willi *et al.* 2021).

*Pomaderris oblongifolia* N.G.Walsh and *P. buchanensis* N.G.Walsh are closely related, highly restricted shrubs occurring in fragmented locations along the Snowy River in East Gippsland, Victoria. Both species were identified as high priority for urgent management intervention following the extensive fires in eastern Australia in 2019/2020 (Gallagher 2020). *Ex situ* conservation, germplasm collection and genetic augmentation has been used in an effort to halt or reverse the decline of several threatened *Pomaderris* spp. (Roberts & Trueman 2016; Chen *et al.* 2019; DELWP 2022). However, seed has been difficult to obtain for many species of *Pomaderris* (McAuliffe *et al.* 2016; Roberts & Trueman 2016) including several triploid species (Chen *et al.* 2019). Polyploidy and/or asexual reproduction via apomixis have been recorded in a substantial number of *Pomaderris* taxa and have been linked with species richness in the genus (Harvey & Rattenbury 1985; Chen *et al*. 2019; Nge *et al.* 2024). In line with flow cytometry measurements by Chen *et al.* (2019), recent genomic work has found *P. oblongifolia* to be diploid ($2n = 24$) (Nge *et al*. 2024). *Pomaderris buchanensis*, which was not assessed by flow cytometry, is suggested to be triploid with this genomic data (Nge *et al*. 2024). Apomixis has been reported for both diploid and higher ploidy levels in *Pomaderris* species and evidence exists for both diploid and tetraploids in the same *Pomaderris* species (Harvey & Rattenbury 1985; Chen *et al*. 2019). While seed production has not been observed in natural populations of *P. oblongifolia* nor *P. buchanensis*, given the isolation of populations, it is unlikely to be observed without targeted trips. Thus, mating systems, seed viability and natural seed banks are unknown for both taxa.

This research provides an assessment of genetic diversity in *P. oblongifolia* and *P. buchanensis* using a proprietary reduced representation sequencing method (DArTseq; Diversity Arrays Technology, Canberra, Australia) to identify localised genetic structure and connectivity among locations. Clonality within locations and relationships between taxa are examined using genomic data to improve conservation assessment and guide *ex situ* germplasm collections of both taxa. Future areas of research are identified to guide conservation of these species in the face of increasing environmental stress.

# Methods

## Study species

*Pomaderris oblongifolia* and *P. buchanensis* are shrubs endemic to the Snowy River in Victoria, southeastern Australia, and are listed as Endangered (EN) and Critically Endangered (CR), respectively, under the *Flora and Fauna Guarantee Act 1988* (Vic). The listings are due to small numbers of plants occurring in disjunct locations within the fire-prone habitat across a narrow length of the Snowy River.

*Pomaderris oblongifolia* is endemic to the gorge tract of the Snowy River in East Gippsland, Victoria, from McKillops Bridge, the Little River Gorge, and other locations downstream to the confluence of the Snowy and Buchan Rivers. *Pomaderris buchanensis* has a narrower distribution, being known historically from only four small, disjunct sites from just north of the Buchan River confluence to Long Point on the Snowy River. *Pomaderris buchanensis* was once considered a hybrid of *P. oblongifolia* and *P. aspera* and is distinguished from *P. oblongifolia* by leaf and floral characters (Walsh 2008). Both *P. oblongifolia* and *P. buchanensis* often co-occur with *P. aspera*, a widespread, common diploid species (Chen *et al.* 2019; Nge *et al.* 2024), which differs from the species of interest in being a tree with much larger leaves and floral features (Walsh 2022). Pollinators, seed dispersal and fire response are not specifically known for either *P. oblongifolia or P. buchanensis*, however, in other *Pomaderris* species, insects are implied as primary pollinators, and positive post-fire germination responses and limited unaided seed dispersal have been found (Patykowski *et al.* 2014; 2016). Most *Pomaderris* species are obligate seeders (Whelan 1995, Le Breton *et al.* 2019) but in some locations of low intensity fire, *P. oblongifolia,* has been observed to resprout basally (DELWP 2022).

The Victorian Conservation Seedbank (VCS) does not hold any seed collections for either target taxon. Plants are cultivated at Royal Botanic Gardens Victoria (RBGV), however, the small amount of seed collected from these cultivated plants is of unknown viability and has the potential to include hybrids due to many *Pomaderris* species being cultivated in close proximity and therefore not representative of the genetic variation of the species.

## Sampling

Surveys for *Pomaderris oblongifolia* and *P. buchanensis* targeted previously recorded locations in the Snowy River National Park based on National Herbarium of Victoria herbarium records, field trips and locations observed along the Snowy River by RBGV staff in January and October 2021. The landscape surveyed

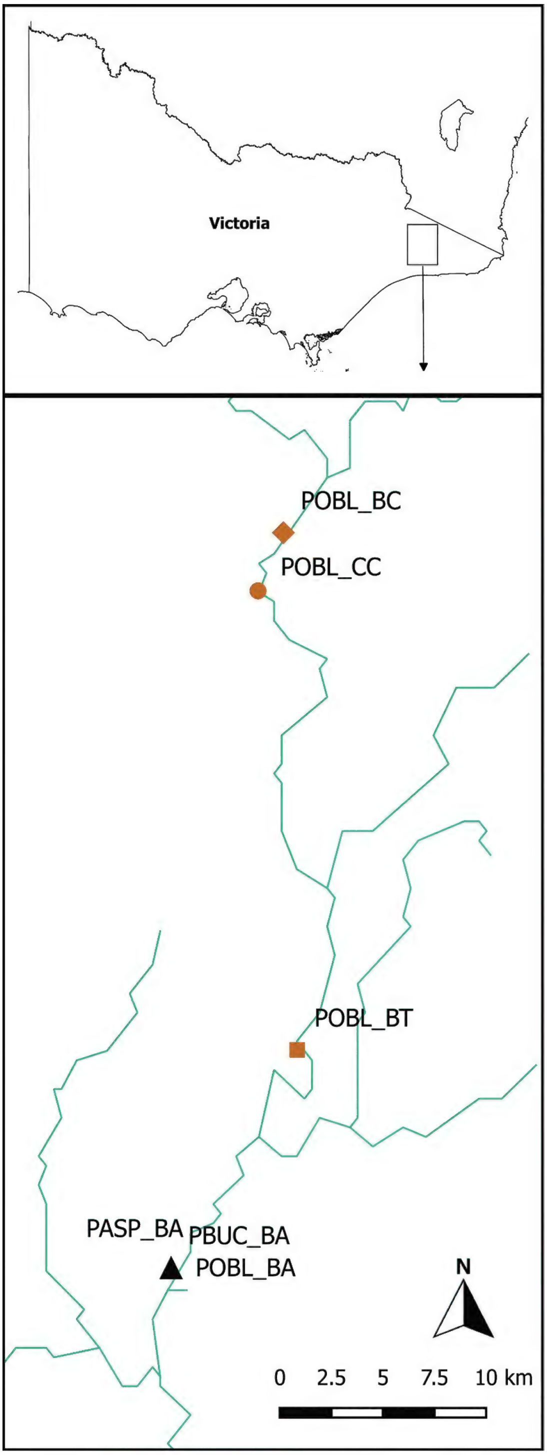

**Figure 1.** Location of sampled populations of *Pomaderris* species in this study. *P. oblongifolia* (POBL), *P. buchanensis* (PBUC), *P. aspera* (PASP). Locations occur along the Snowy River in East Gippsland in the southeast corner of mainland Australia.

included steep, rocky banks and slopes, or rocky areas with crevices where plants were emerging post-fire. Survival and regeneration were confirmed for *P. oblongifolia* at four locations while *P. buchanensis* was found at two locations (MEL collections NGW 9170/ PBUC_BA and NGW 9178) where it co-occurred with *P. aspera*. Samples of *P. buchanensis* were collected at only one of these locations (Figure 1, Table 1). The entire field survey area had burnt at varying levels of intensity in the Black Summer bushfires (2019–2020), with some small patches of unburnt remnant vegetation. Most plants sampled were 'sub-adult' non-reproductive one year old plants. Twenty-three samples of *P. oblongifolia* were collected from the four locations along the Snowy River (Figure 1) and encompass the geographic range of the species: near the confluence with Boundary Creek (POBL_BC, 5 samples, unburnt adults), near the confluence with Currie Creek (POBL_CC, 5 samples, fire severity not recorded), at New Guinea Bend / Betts Creek (POBL_BT, 5 samples, moderate burn, basal resprout) and near Basin Creek Falls (POBL_BA, 8 samples, unburnt adults). Twenty-one samples of *P. buchanensis* were collected from a site near Basin Creek Falls (NGW 9170; PBUC_BA) which was located on the opposite riverbank from POBL_BA. The site was unburnt and consisted of c. 200 flowering plants approximately 150 cm high (DELWP 2022). Four samples of *P. aspera* co-occurring with *P. buchanensis* were collected at Snowy River Basin Creek (PASP_BA). Sampling was spread across the distribution of the target taxon at each location, with samples collected approximately 5 m apart.

## DNA extraction, amplification and sequencing

Approximately 15 mg from each of the 48 leaf samples plus one technical replicate were sent to a commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq™ analysis which is a reduced representation sequencing method (Sansaloni *et al.* 2010; Kilian *et al.* 2012; Cruz *et al.* 2013). DNA was extracted at DArT using the Nucleo Mag Plant C-Bead kit (Machery Nagel, Germany) on a Tecan 100 platform per the manufacturer's protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and MseI and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised

**Table 1. Sites sampled for *Pomaderris* along the Snowy River in Victoria. *P. oblongifolia* (POBL), *P. buchanensis* (PBUC), *P. aspera* (PASP). Number of samples collected at each location with number of unique genotypes detected in parentheses. Accession number in the National Herbarium of Victoria (MEL) collection.**

| Site Code | No. of samples (unique genotypes) | Location | MEL Accession no. |
|---|---|---|---|
| POBL_BA | 8 (1) | Snowy River, 200 m from gauging station, upstream of Basin Creek (BA) | MEL 2494342A |
| PASP_BA | 4 (1) | Snowy River, 200 m from gauging station, upstream of Basin Creek (BA) | MEL 2494339A |
| PBUC_BA | 21 (1) | Snowy River, 200 m from gauging station, upstream of Basin Creek (BA) | MEL 2496564A |
| POBL_CC | 5 (1) | Junction of Currie Creek and Snowy River (CC) | MEL 2494325A |
| POBL_BT | 5 (1) | South of Betts Creek Track on New Guinea Bend (BT) | MEL 2496543A |
| POBL_BC | 5 (3) | North of the junction with Boundary Creek (BC) | MEL 2496517A |

and pooled for sequencing in a single lane of an Illumina HiSeq2500. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT's proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DArTsoft14).

## Data analysis

All analyses were undertaken using packages implemented in R ver. 4.2.1 (R Core Team 2022) and RStudio ver. 2023.12.1+402.pro1 (R Studio Team 2023). Raw unfiltered DNA SNP data derived from DArTSeq™ analysis comprised 49 samples across the three taxa and 27686 loci including one technical replicate. Data for *P. oblongifolia*, *P. buchanensis* and *P. aspera* were analysed together to enable comparison between the taxa. Data were stringently filtered to a locus rate of 90% and individual call rate of 75%, a reproducibility score of 1, a minor allele frequency (MAF) of 8%, a Hardy-Weinberg equilibrium with a 5% level of significance, secondaries and monomorphs removed and finally filtered on Hamming distance to remove potential paralogues using the package dartR (Gruber *et al.* 2018). This resulted in a filtered dataset of 3939 SNPs across 49 samples.

To identify clonal genotypes, a pairwise genetic distance (Euclidian) was calculated between all samples using the R package dartR (Gruber et al. 2018). A 'sequencing error' was calculated by taking the pairwise genetic distance between the identical samples (technical replicate, value = 30.46). Pairwise samples with less than twice the average sequencing error (distance value ≤60.92) were considered ramets of a clonal genotype. The mean distance between clonal samples (ramets) of 36.98 (±5.15 S.D.) was clearly distinct from the mean distance among all other stems (134.86 ± 34.59 S.D.), giving high confidence in clone identification.

The technical repeat was then removed, and data refiltered as described above, with MAF set to 5%. To verify the relationship of clonal stems, pairwise kinships were estimated on the filtered dataset of 3948 loci across 48 samples using PLINK method of moment (MoM) for the Identity-By-Descent (IBD) Analysis in SNPRelate (snpgdsIBDMoM; Zheng *et al*. 2012). While a conservative method (McMaster et al. 2024), it is ideal for non-model species. Kinship values of 0.50 represent a monozygotic twin, with a kinship threshold of $\phi=0.354$ used to define clones. Kinship values of $\phi=0.25$ and higher indicate a first-degree relationship (full siblings, parent-child etc.) and $\phi=0.125\text{-}0.25$ indicate a second-degree relationship (i.e., half-siblings, grandparent-grandchild etc). Unrelated pairs are expected to have values close to $\phi=0$ (Manichaikul *et al*. 2010). Kinship values were presented separately for *P. oblongifolia* as the analysis found no relationship between this taxon and *P. buchanensis* and *P. aspera*.

Finally, the dataset was refined to include only one sample per clonal group and was comprised of 16136 SNPs across 8 samples. Data were then filtered as above with MAF set to 12.5%. Principal component analysis (PCA) was undertaken to identify genetic clusters and visualise the major axes of variation between clusters using adegenet (Jombart 2008; Jombart & Ahmed 2011). Individual observed heterozygosity and inbreeding coefficients were assessed using hierfstat (Goudet & Jombart 2020) and dartR (Gruber *et al.* 2018).

Isolation by distance and fixed differences between locations were assessed using dartR (Gruber *et al.* 2018). The R package ggplot2 (Wickham 2016) was used to visualise results.

## Results

Clonality was evident in all sampled locations of *Pomaderris oblongifolia, P. buchanensis* and *P. aspera* (Tables 2 & 3, Supplementary Table 1 & 2 available at www.rbg.vic.gov.au/science/journal/). Kinship analysis demonstrated that three locations of *P. oblongifolia* POBL_BT, POBL_CC and POBL_BA are monoclonal with a unique genotype at each site (Tables 1 & 2). At the fourth population, POBL_BC, three genotypes were identified with one recovered from three of the five samples collected. The location with both *P. aspera* and *P. buchanensis* had two genotypes, broadly corresponding to the morphological taxon identification at the time of sampling (Table 1). Importantly, *P. buchanensis* is closely related to *P. aspera* but not to *P. oblongifolia* (Tables 3 & 5; Supplementary Table 1 & 2).

For *P. oblongifolia*, the genotype at POBL_BT and sample POBL_0192 from Boundary Creek have a kinship value of 0.20 (Table 2) which identifies them as second-degree relatives such as half-sibs and close to the value of 0.25 which defines first degree relationships such as parent-offspring and full-sibs. Similarly, the genotype at POBL_CC has a third-degree relationship with all three Boundary Creek POBL_BC genotypes (Table 2). This result and the increasing genetic distance with POBL_BC is consistent with POBL_BC at the north of the species' distribution being a potential 'source' of populations founded further down the catchment and an indicator of historical connectivity (Figure 1).

At Basin Creek, the kinship values for *P. aspera* indicate that the samples collected are monoclonal and that one individual, PBUC_0290, collected as *P. buchanensis*, is in fact a clonal genotype of the *P. aspera* samples collected from this location (Table 3). The remaining 20 samples of *P. buchanensis* were also identified as being monoclonal. It should be noted that the average kinship values are lower between individuals PBUC_0301 and PBUC_0302 (bold) and all other samples, and while lower than the other values, they are still above the clone threshold. A single kinship pair value of $\phi$=0.33 between PBUC_0289 and PBUC_0301 was found suggesting that these individuals have a first-degree relationship, such as parent-offspring or full sibling rather than being clonal, however this value is marginal. Of note, is the first-degree relationship between the *P. aspera* and *P. buchanensis* genotypes, indicating a parent-offspring or full sibling relationship between the taxa. The exceptions are the kinship values for samples PBUC_0301 ($\phi$=0.09-0.10) and PBUC_0302 ($\phi$=0.13-0.14) which suggest a second-degree relationship (i.e. half-siblings, grandparent-grandchild etc).

The final dataset, representing one sample per clonal group, contained eight samples and 3049 loci after filtering across four *P. oblongifolia* locations and the *P. buchanensis*–*P. aspera* location. Overall, nuclear genetic diversity was 0.232, with observed heterozygosity levels ranging from 0.182 to 0.255 (Table 4). Inbreeding ($F_{IS}$) values ranged 0.346 for PASP_BA to 0.533 for PBUC_BA (Table 4).

Pairwise comparisons of fixed differences ranged from 410–426 for *P. oblongifolia* individuals within the same population (POBL_BC; POBL_0190, POBL_0191, POBL_192; Table 5). Fixed differences between some *P. oblongifolia* populations were lower. For example, the fixed differences between POBL_CC and POBL_BC ranged from 319-398 and POBL_0192 from Boundary Creek (BC) and POBL_0090 from Betts Track (BT) had even lower fixed differences, reflecting the close relationship identified in the kinship analysis (Tables 2 & 5). Fixed differences were highest between PASP_BA, PBUC_BA and all *P. oblongifolia* ranging from 709-1013 and were lowest (2) between PASP_BA and PBUC_BA (Table 5).

The principal components analysis showed high levels of differentiation among samples, with all *P. oblongifolia* samples and the *P. buchanensis*–*P. aspera* samples separated across the first axis which accounts for 43.32% of the variation (Figure 2). The *P. aspera* sample PASP_0280 and *P. buchanensis* sample PBUC_0294 are closely clustered in the negative space of axis 1. The *P. oblongifolia* samples are spread across the second axis (14.32% of the variation) with clustering of individuals that are more closely related (Figure 2). The proximate placement of POBL_BC individual (POBL_0192) with the genotype from POBL_BT (POBL_0090) and POBL_BC individual (POBL_0191), with the POBL_CC (POBL_0050) genotype again reflects the close relationship between these sites (Figure 2, Table 2).

**Table 2. Pairwise kinship values for *Pomaderris oblongifolia* samples within and among populations. Darker shading indicates more closely related individuals. Underline indicates samples used to represent the unique individuals in genetic diversity statistics.**

| Pop | | BA<br>POBL | | | | | | | | CC<br>POBL | | | | | BC<br>POBL | | | | | BT<br>POBL | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Sample** | **0271** | **0278** | **0277** | **0276** | **0275** | **0274** | **0273** | **0272** | **0046** | **0047** | **0048** | **0049** | **0050** | **0190** | **0191** | **0192** | **0188** | **0189** | **0088** | **0089** | **0090** | **0091** | **0092** |
| **BA<br>POBL** | **0271** | | | | | | | | | | | | | | | | | | | | | | | |
| | **0278** | 0.44 | | | | | | | | | | | | | | | | | | | | | | |
| | **0277** | 0.44 | 0.44 | | | | | | | | | | | | | | | | | | | | | |
| | **0276** | 0.43 | 0.43 | 0.44 | | | | | | | | | | | | | | | | | | | | |
| | **0275** | 0.42 | 0.43 | 0.42 | 0.42 | | | | | | | | | | | | | | | | | | | |
| | **0274** | 0.43 | 0.43 | 0.43 | 0.43 | 0.42 | | | | | | | | | | | | | | | | | | |
| | **0273** | 0.44 | 0.43 | 0.44 | 0.43 | 0.42 | 0.43 | | | | | | | | | | | | | | | | | |
| | **0272** | 0.41 | 0.41 | 0.40 | 0.41 | 0.40 | 0.40 | 0.41 | | | | | | | | | | | | | | | | |
| **CC<br>POBL** | **0046** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | | | | | |
| | **0047** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.45 | | | | | | | | | | | | | | |
| | **0048** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.45 | 0.45 | | | | | | | | | | | | | |
| | **0049** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.45 | 0.45 | 0.44 | | | | | | | | | | | | |
| | **0050** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.44 | 0.44 | 0.44 | 0.44 | | | | | | | | | | | |
| **BC<br>POBL** | **0190** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | | | | | | | | | | |
| | **0191** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.03 | | | | | | | | | |
| | **0192** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.00 | 0.03 | | | | | | | | |
| | **0188** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0.03 | 0.02 | 0.02 | 0.02 | 0.45 | 0.03 | 0.01 | | | | | | | |
| | **0189** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.44 | 0.03 | 0.00 | 0.44 | | | | | | |
| **BT<br>POBL** | **0088** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.0 | 0.20 | 0 | 0 | | | | | |
| | **0089** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.21 | 0 | 0 | 0.44 | | | | |
| | **0090** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.19 | 0 | 0 | 0.42 | 0.42 | | | |
| | **0091** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.20 | 0 | 0 | 0.44 | 0.44 | 0.42 | | |
| | **0092** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.20 | 0 | 0 | 0.44 | 0.43 | 0.41 | 0.44 | |

**Table 3. Pairwise kinship values for *Pomaderris buchanensis and P. aspera* samples from the Basin Creek population. Darker shading indicates more closely related individuals. Underline indicates samples used to represent the unique individuals in genetic diversity statistics.**

| | | PASP | | | | PBUC | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **0279** | **0282** | **0281** | **0280** | **0303** | **0295** | **0287** | **0302** | **0294** | **0286** | **0301** | **0293** | **0285** | **0300** | **0292** | **0284** | **0299** | **0291** | **0283** | **0298** | **0290** | **0297** | **0289** | **0296** | **0288** |
| **PASP** | **0279** | | | | | | | | | | | | | | | | | | | | | | | | | |
| | **0282** | 0.49 | | | | | | | | | | | | | | | | | | | | | | | | |
| | **0281** | 0.49 | 0.50 | | | | | | | | | | | | | | | | | | | | | | | |
| | **0280** | 0.49 | 0.50 | 0.50 | | | | | | | | | | | | | | | | | | | | | | |
| **PBUC** | **0303** | 0.19 | 0.20 | 0.20 | 0.20 | | | | | | | | | | | | | | | | | | | | | |
| | **0295** | 0.19 | 0.20 | 0.20 | 0.20 | 0.47 | | | | | | | | | | | | | | | | | | | | |
| | **0287** | 0.22 | 0.23 | 0.22 | 0.23 | 0.47 | 0.48 | | | | | | | | | | | | | | | | | | | |
| | **0302** | 0.13 | 0.14 | 0.14 | 0.14 | 0.40 | 0.41 | 0.43 | | | | | | | | | | | | | | | | | | |
| | **0294** | 0.21 | 0.23 | 0.22 | 0.22 | 0.48 | 0.48 | 0.50 | 0.43 | | | | | | | | | | | | | | | | | |
| | **0286** | 0.22 | 0.23 | 0.23 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | | | | | | | | | | | | | | | | |
| | **0301** | 0.09 | 0.10 | 0.10 | 0.10 | 0.38 | 0.39 | 0.41 | 0.36 | 0.41 | 0.41 | | | | | | | | | | | | | | | |
| | **0293** | 0.22 | 0.23 | 0.22 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.42 | | | | | | | | | | | | | | |
| | **0285** | 0.21 | 0.22 | 0.22 | 0.22 | 0.47 | 0.48 | 0.50 | 0.43 | 0.49 | 0.50 | 0.42 | 0.50 | | | | | | | | | | | | | |
| | **0300** | 0.20 | 0.21 | 0.21 | 0.21 | 0.46 | 0.47 | 0.48 | 0.42 | 0.48 | 0.48 | 0.39 | 0.48 | 0.48 | | | | | | | | | | | | |
| | **0292** | 0.22 | 0.23 | 0.22 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.42 | 0.50 | 0.50 | 0.48 | | | | | | | | | | | |
| | **0284** | 0.22 | 0.23 | 0.23 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.42 | 0.50 | 0.50 | 0.48 | 0.50 | | | | | | | | | | |
| | **0299** | 0.20 | 0.21 | 0.21 | 0.22 | 0.46 | 0.47 | 0.49 | 0.41 | 0.49 | 0.49 | 0.40 | 0.49 | 0.48 | 0.47 | 0.49 | 0.49 | | | | | | | | | |
| | **0291** | 0.22 | 0.23 | 0.22 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.41 | 0.50 | 0.49 | 0.48 | 0.50 | 0.50 | 0.48 | | | | | | | | |
| | **0283** | 0.22 | 0.23 | 0.22 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.42 | 0.50 | 0.50 | 0.48 | 0.50 | 0.50 | 0.49 | 0.50 | | | | | | | |
| | **0298** | 0.21 | 0.22 | 0.22 | 0.22 | 0.47 | 0.48 | 0.49 | 0.43 | 0.49 | 0.49 | 0.41 | 0.49 | 0.49 | 0.48 | 0.49 | 0.49 | 0.48 | 0.49 | 0.49 | | | | | | |
| | **0290** | 0.49 | 0.50 | 0.50 | 0.50 | 0.20 | 0.20 | 0.23 | 0.14 | 0.23 | 0.23 | 0.10 | 0.23 | 0.22 | 0.21 | 0.23 | 0.23 | 0.21 | 0.23 | 0.23 | 0.22 | | | | | |
| | **0297** | 0.21 | 0.22 | 0.22 | 0.22 | 0.45 | 0.47 | 0.48 | 0.42 | 0.48 | 0.48 | 0.39 | 0.48 | 0.48 | 0.46 | 0.48 | 0.48 | 0.47 | 0.48 | 0.48 | 0.47 | 0.22 | | | | |
| | **0289** | 0.27 | 0.27 | 0.27 | 0.27 | 0.39 | 0.40 | 0.42 | 0.35 | 0.41 | 0.42 | 0.33 | 0.42 | 0.41 | 0.40 | 0.41 | 0.42 | 0.40 | 0.41 | 0.42 | 0.41 | 0.27 | 0.41 | | | |
| | **0296** | 0.22 | 0.23 | 0.23 | 0.23 | 0.46 | 0.47 | 0.49 | 0.42 | 0.48 | 0.49 | 0.40 | 0.49 | 0.48 | 0.47 | 0.49 | 0.49 | 0.47 | 0.49 | 0.49 | 0.48 | 0.23 | 0.47 | 0.41 | | |
| | **0288** | 0.22 | 0.23 | 0.23 | 0.23 | 0.47 | 0.48 | 0.50 | 0.43 | 0.50 | 0.50 | 0.42 | 0.50 | 0.50 | 0.48 | 0.50 | 0.50 | 0.49 | 0.50 | 0.50 | 0.49 | 0.23 | 0.48 | 0.42 | 0.49 | |

**Table 4. Genetic diversity characteristics of *Pomaderris oblongifolia, P. buchanensis* and *P. aspera* individuals.**

| Taxon | Population | Individual | Observed heterozygosity ($H_o$) | Inbreeding coefficient ($F_{IS}$) |
|---|---|---|---|---|
| PASP | BA | 0280 | 0.244 | 0.376 |
| PBUC | BA | 0294 | 0.182 | 0.533 |
| POBL | BA | 0271 | 0.217 | 0.445 |
|  | CC | 0050 | 0.229 | 0.414 |
|  | BT | 0090 | 0.243 | 0.377 |
|  | BC | 0190 | 0.242 | 0.379 |
|  |  | 0191 | 0.240 | 0.385 |
|  |  | 0192 | 0.255 | 0.346 |

**Table 5. Matrix of fixed differences among individuals of *Pomaderris oblongifolia, P. buchanensis* and *P. aspera.***

|  |  | BA | BA | CC | BT | BC |  |  | BA |
|---|---|---|---|---|---|---|---|---|---|
|  |  | **PASP_0280** | **PBUC_0294** | **POBL_0050** | **POBL_0090** | **POBL_0190** | **POBL_0191** | **POBL_0192** | **POBL_0271** |
| **BA** | **PASP_0280** | 0 |  |  |  |  |  |  |  |
| **BA** | **PBUC_0294** | 2 | 0 |  |  |  |  |  |  |
| **CC** | **POBL_0050** | 709 | 751 | 0 |  |  |  |  |  |
| **BT** | **POBL_0090** | 897 | 961 | 440 | 0 |  |  |  |  |
| **BC** | **POBL_0190** | 731 | 777 | 376 | 461 | 0 |  |  |  |
|  | **POBL_0191** | 604 | 648 | 319 | 462 | 410 | 0 |  |  |
|  | **POBL_0192** | 844 | 912 | 398 | 109 | 426 | 419 | 0 |  |
| **BA** | **POBL_0271** | 944 | 1013 | 553 | 541 | 518 | 547 | 501 | 0 |

## Discussion

The two major findings of this study were that clonality was evident in all sampled locations of *Pomaderris oblongifolia, P. buchanensis* and *P. aspera*, and that *P. buchanensis* has a close relationship with *P. aspera* but not with *P. oblongifolia*. The use of SNPs generated by DArTseq allowed an evaluation of genomic diversity within *P. oblongifolia* and *P. buchanensis*. The apparent recovery of *P. oblongifolia* and *P. buchanensis* following the widespread bushfires in 2019/2020 is evidenced in the recruitment of seedlings at affected locations. The discovery of extensive clonality in both species stresses the importance of understanding the levels and patterns of genetic diversity across a species range for *in situ* and *ex situ* conservation. These results raise uncertainty about the level of sexual reproduction and highlight the knowledge gaps in the breeding system.

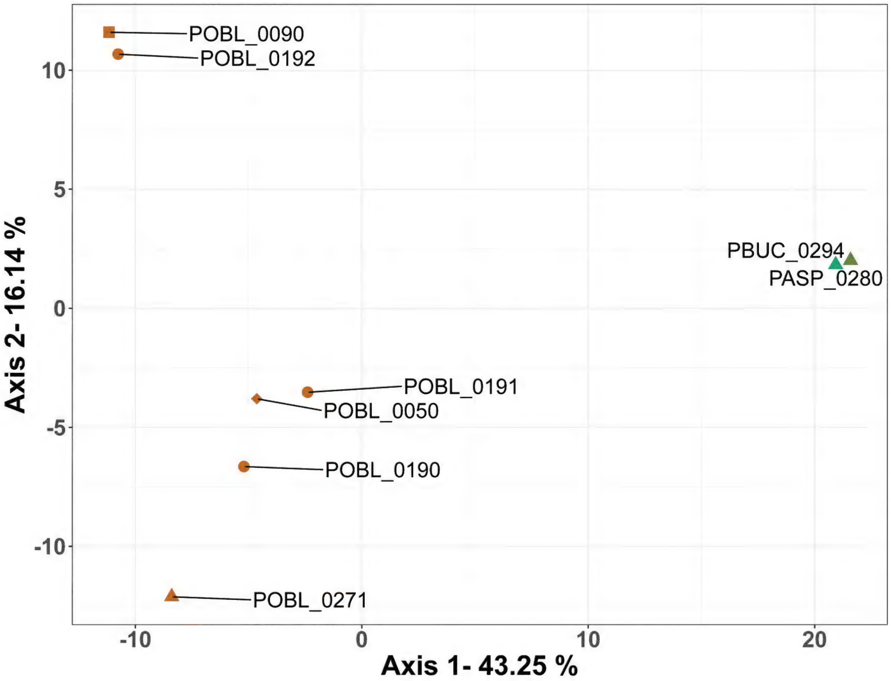

**Figure 2.** Principal component analysis of genetic differentiation in *Pomaderris oblongifolia* (squares, POBL), *P. buchanensis* (triangle, PBUC) and *P. aspera* (circles, PASP) on the Snowy River.

## Population biology of the rare Snowy River *Pomaderris*

The identification of six *Pomaderris oblongifolia* genotypes from 23 samples and the occurrence of three monoclonal sites significantly reduces the number of individuals for conservation assessment purposes (TSSC 2021; IUCN SPC 2022). The presence of three genotypes within the *P. oblongifolia* Boundary Creek location (POBL_BC) suggests that some level of sexual reproduction has occurred in the past. Plants at this site were recorded as unburnt mature plants, occurring at a lower density and over a much wider area (100 individuals across 5000 m$^2$) compared to other locations (50–100 individuals across 2500 m$^2$) which may indicate different disturbance histories. The high levels of clonality observed in *P. oblongifolia* reduce the number of genetically unique individuals to considerably less (20–60%) than the population census counts.

Even more critical is the result for *P. buchanensis,* with only one genetically unique individual recovered from 21 samples; if the same genetic patterns exist in the other known locations, this could mean only two to four unique genotypes of *P. buchanensis* exist for the species. The close relationship between samples collected as *P. buchanensis* and *P. aspera* at Basin Creek also raises questions around the delimitation of these species. The similarity between *P. buchanensis* and *P. aspera* has important conservation implications. *Pomaderris aspera* is widespread across the east coast of Australia and has a close affinity to both *P. oblongifolia* and *P. buchanensis* with the three species thought to have speciated less than 5 Mya (Nge *et al*. 2021). *Pomaderris aspera* is known to co-occur with *P. oblongifolia* and *P. buchanensis* at a few locations along the Snowy River (Walsh 2008). The kinship analyses in this study identified monoclonality in both the *P. buchanensis* and *P. aspera* samples from the Basin Creek. A first-degree relationship (parent-offspring or full sibling) was also identified between the *P. buchanensis* and *P. aspera* samples but the *P. buchanensis* samples did not have the same level of genetic similarity to *P. oblongifolia* (Supplementary Table 1). Based on this data, there is no evidence that *P. buchanensis* is a contemporary hybrid between *P. aspera* and *P. oblongifolia* but does not discount both species being implicated in the evolution of *P. buchanensis* in more ancient time scales, given their close phylogenetic relatedness.

## Life history characteristics that may cause low genetic diversity

Diversification in *Pomaderris* is thought to consist largely of *in situ* speciation within individual biomes, potentially driven by high levels of polyploidy, apomixis, an ability to resprout and sexual production of seed, all of which allow for rapid colonisation of new habitat (Nge *et al.* 2021; 2024). No seed set has been observed in *P. buchanensis* either in the wild or in cultivation which adds to the recent evidence that the species is a triploid (Nge *et al*. 2024), a characteristic found in other rare *Pomaderris* (*P. pallida, P. reperta*; Chen *et al*. 2019). While polyploidy may provide advantageous traits for survival, triploids may be partially or fully sterile due to chromosome mismatching (Comai 2005; Chen et al. 2019), an obstacle that can be overcome to some extent by asexual reproduction. The misidentification of one *P. aspera* individual as *P. buchanensis* (PBUC_0290) highlights their morphological similarity, supports their close phylogenetic relatedness (Nge *et al.* 2021; 2024) and offers an insight into the evolutionary history of *P. buchanensis*. Previous studies have indicated that *P. oblongifolia* and *P. aspera* are diploid, but intraspecific ploidy variation has been found in other species of *Pomaderris* (Chen *et al*. 2019). Triploids are often found in mixed-ploidy populations (Husband 2004; Wallace *et al*. 2017). A limitation of this study was the absence of samples from an additional *P. buchanensis* location and broader sampling of *P. aspera* from the Snowy River. Further surveys, more thorough sampling, and the inclusion of samples of all three taxa (*P. buchanensis, P. aspera* and *P. oblongifolia*) from the Snowy River in future genetic studies, coupled with flow cytometry across multiple individuals at multiple sites, are needed to characterise ploidy levels. Better sampling and other sequencing approaches would also allow for genomic estimation of ploidy using nQuire as described in Nge *et al.* (2024) or ploidyNGS (Augusto Corrêa Dos Santos *et al.* 2017). In combination, these techniques could elucidate the evolutionary relationship between the taxa and whether *P. buchanensis* is a triploid variant derived directly from *P. aspera*.

While patterns of low genetic diversity and population structure could result from a dramatic loss of individuals in a species with low genetic variation, asexual reproduction is a more likely explanation given the life history characteristics previously recorded for *Pomaderris*. Basal resprouting has been observed in *P. oblongifolia*, however vegetative reproduction via root-suckering has not been observed in either that taxon or *P. buchanensis*. Apomixis is a possible explanation for the clonality and low genetic diversity identified in our data that reportedly occurs in diploid *Pomaderris* species in southeastern Australia (Chen *et al*. 2019) as well as many polyploid species (Bicknell and Koltunow 2004; Hojsgaard & Hörandl 2019; reviewed in Hörandl *et al*. 2024). Given the level of clonality and the apparent recruitment from seed after fire, an understanding of the realised mating system in *P. oblongifolia* and *P. buchanensis* is needed to inform appropriate conservation actions. If apomixis is the most likely explanation, its presence needs to be confirmed with embryological studies and the evaluation of endosperm:embryo ploidy levels if seed can be obtained. If present, apomixis must be quantified across the distribution of both taxa to ensure that the likelihood of collecting apomictic seed from wild populations is understood. If seed is collected for *ex situ* conservation purposes prior to reproductive biology studies, it is particularly important to follow best practice by storing seed lots as separate maternal lines (Commander et al. 2018) as separate plants may be clonemates producing genetically identical seeds. If possible, germinants from any reproductive biology studies should be included in future genetic studies.

### Conservation of both taxa

*In situ* seedbanks play an important role in conserving the genetic diversity of a species in fire prone environments and sexually-produced seed enhances the genotypic diversity at a location. The length of time to flowering has not been recorded post-fire for either *P. oblongifolia* or *P. buchanensis* but is needed for planning fire regimes with time intervals long enough for several years of seed to accumulate in the seedbank or to assess the potential seedbank following wildfires. Re-assessment of the genetic structure of both narrow-ranged endemic *Pomaderris* species in this study over time is advisable to follow the genetic demographics and guide germplasm sampling to effectively maintain the genetic representation of wild populations in *ex situ* collections. It is prudent to propagate individuals vegetatively from formally identified, wild collected and genotyped individuals which would enable replication in *ex situ* experiments to characterise the breeding system. Given the small percentage of plants analysed at each site, additional, dense sampling is advisable to confirm levels of clonality. The discovery of additional new genotypes will be of conservation value, particularly if *ex situ* collections are to be used for seed production.

Evaluation of the extinction risk of *P. oblongifolia* and *P. buchanensis* against IUCN criteria, based on the combination of the census of mature genetic individuals through population sub-sampling and geographic range size, shows that both taxa warrant listing under the CR threat category. To ensure that the conservation management applied to *P. oblongifolia* and *P. buchanensis* is appropriate, it is vital to determine their breeding systems. Episodic sexual recruitment is known to occur in plants capable of asexual reproduction but can be difficult to confirm directly (Eriksson 1989) as apomictic seedlings in natural populations are likely to be indistinguishable from sexually produced seed in the absence of genetic testing. Breeding system studies would also be important for resolving the similarity between *P. buchanensis* and *P. aspera*. Equally, if *P. buchanensis* is a variant derived directly from *P. aspera*, it may be a candidate for removal from the FFG/EPBC listing and managed with *P. aspera* as a taxon of least concern.

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## Supplementary information

1. Filtered matrix of pairwise genetic distance, on the filtered dataset of 3939 loci across 49 samples, including technical replicate (italicised).
2. Pairwise kinship values (ϕ) on the filtered dataset of 3948 loci across 48 samples using the KING method of moments for identity-by-descent (IBD).

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Muelleria

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# Pulling the wool over our eyes: deciphering genomic variation in *Acacia lucasii* (Fabaceae) and close allies

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## Introduction

Several regions of Australia have high numbers of unique, restricted and rare flora species due to a combination of environmental factors including temperature and precipitation, soil texture, and topographic heterogeneity (Mokany *et al.* 2022). Southeastern Australia is species-rich and diverse owing to the complex profile of the Great Dividing Range that provides substantial geological, elevation, climatic and environmental complexity (Taylor 1994, Ollier 1995) which may have influenced the expansion or contraction of species' ranges during historic climatic oscillations (Bryant & Krosch 2016, Byrne 2008). The combination of landscape-wide environmental factors and environmental events play a role in shaping the species diversity and distributions seen in different plant groups common in the southeast (e.g. Rosetto *et al.* 2012, Nge *et al.* 2021, McMaster *et al.* 2024).

The complex patterns seen in the diversity and distributions of taxa make evaluation of the genetic variation, structure and historical gene flow across a species' range an important step in defining conservation units and can aid in the identification of cryptic taxa. Genetic evaluation prior to conservation actions enables the use of species-specific information to guide management. Genetically informed collection and use of germplasm *in situ* and in the establishment of seed orchards

## Abstract

*Acacia lucasii* Blakely (woolly-bear wattle) occurs in only two remote, disjunct localities in southeast Australia. The entire species' range was burnt in the Black Summer megafires (2019–2020). This study investigates the genomic variation across the geographic range of *A. lucasii*, the taxonomically associated *A. costiniana* Tindale and a closely related population of uncertain status. High levels of genetic differentiation were found between all locations and taxa. *Acacia lucasii* populations had low genetic diversity and high levels of inbreeding, reflecting the isolation of populations that are likely remnants of a previously more widespread precursor affected by historic climatic oscillations resulting in contemporary geographic separation and divergence. Irrespective of taxon, the genetically distinct populations may be best conserved as individual conservation units.

***Keywords:*** DArTseq; *Acacia*; species complex; range disjunction, restricted range

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or the design of translocations (Commander *et al.* 2018) lessens the risk of inappropriate genetic combinations (Coates *et al.* 2018). This approach should facilitate the creation of viable, self-sustaining populations with the genetic integrity to withstand and adapt to future environmental changes (Sgrò *et al.* 2011). Range-restricted species and/or those with small populations are particularly susceptible to abiotic stochastic events such as fires and climate change. Such species whose distribution and/or population numbers are severely compromised confound the assumptions of population genetic analysis; that is, sufficiently large population size, the occurrence of random mating, and the absence of mutation, genetic drift, or natural selection (Frankham *et al*. 2002) but are frequently conservation targets. For such range-restricted species, or species complexes where similar species occur in adjacent areas, genetic analysis can be of use in determining the boundaries between taxonomic units, populations, and conservation units, resolving genetic lineages where phylogenies may have been unsuccessful in describing relationships (Coates *et al.* 2018).

The catastrophic Australian megafires in the 2019–2020 bushfire season burnt over 10 million hectares of the landscape and affected some part of the geographic range of between 36–69% of all Australian plant species, including large areas of southeastern Australia (Gallagher *et al.* 2021). Rapid risk assessment determined that 688 Victorian flora species listed on the state *Flora and Fauna Guarantee Act 1988* (Vic) had some portion of their range burnt (DELWP 2022). Plant taxa considered to be most at risk of impacts and extinction from the fires were assessed and prioritised continent-wide, based on their risk of post-fire decline against 11 criteria based on key species traits and impact area (Gallagher *et al.* 2021, Auld *et al.* 2022). A total of 486 flora species were recognised federally as requiring actions to assess impacts and support recovery (DAWE 2020). Among the immediate to medium-term actions proposed, germplasm collection and carefully planned translocations were some of the actions identified as most needed for plant species (DAWE 2020).

The genus *Acacia* Mill. is comprised of 1,082 species with 1,073 mostly endemic species found in Australia where they grow in a wide range of habitats on the mainland, Tasmania, and nearby islands, ranging from coastal to subalpine, tropical, and arid (Kodela & Maslin 2025). Despite the continent-wide distribution of the genus, many species have restricted ranges and disjunct distributions which can elevate their extinction risk (e.g. *Acacia whibleyana* R.S.Cowan & Maslin (Blyth *et al.* 2020), *A. purpureapetala* F.M.Bailey (van der Merwe *et al.* 2021)) often in combination with reduced fecundity and clonality (e.g. *Acacia anomala* C.A.Gardner ex Court (Coates 1988), *A. daviesii* Bartolome (Bartolome *et al.* 2002), *A. sporadica* N.G.Walsh (Walsh 2004)). Many threatened taxa are listed under state and federal legislation due to small population size, range restriction, or occurrence at a single or few disjunct locations (Category B-D, IUCN/SPC 2022). *Acacia lucasii* Blakely (woolly-bear wattle) is a species known from only two remote, disjunct localities in southeastern Australia, namely the Nunniong Plateau in Alpine National Park, Victoria (Vic) and 170 km away in the Upper Turross area in Wadbilliga National Park, New South Wales (NSW) (VicFlora 2025, Maslin 2012a).

*Acacia lucasii* is considered Critically Endangered under the *Flora and Fauna Guarantee Act 1988* (Vic) due to its restricted geographic range in Victoria but has no formal listing in either NSW or federally. As an obligate seeder, fire is likely to stimulate the regeneration of *A. lucasii* seeds stored in the soil seed bank, however, the small number of populations and disjunction between the two localities flag susceptibility to stochastic events and inbreeding as inherent vulnerabilities for the species. The entire range of *A. lucasii* was burnt in the Black Summer megafires and thus the species was one of 31 *Acacia* species that were prioritised for post-fire assessments and recovery actions. Another localised taxon, *A. costiniana* Tindale from the Tinderry Mountains NSW, is taxonomically associated with *A. lucasii*. In addition, herbarium specimens collected from the Nalbaugh Plateau in southeast NSW between 1985 and 2021 determined as either *A. lucasii* or *A. costiniana* may constitute a distinct taxon, based on morphological differences (D. Albrecht, pers. observ.), and is herein referred to as *Acacia* aff. *lucasii*. Known occurrences of the three entities are within protected areas (VicFlora 2025; Maslin 2012a) but among the post-fire response actions proposed for *A. lucasii* are germplasm collection and planned translocations (DAWE 2020), both of which would benefit from the availability of genetic information to guide species-appropriate use of germplasm. In this study, we investigate genomic variation across the geographic range of *A. lucasii* and compare *A. lucasii* to taxonomically associated *A. costiniana* and *A.* aff. *lucasii* to guide conservation actions. We aimed to determine the genetic identity of the putative taxon *A.* aff. *lucasii*, thereby allowing for both future taxonomic revisions and a reliable basis for future species management.

## Methods

### Study species

*Acacia lucasii* grows as an understorey shrub to 4 m high in eucalypt woodland or forest and in heath on exposed rocky granite or quartzite slopes including scree slopes at elevations from 900–1300 m. The species is very distinctive due to its rusty-brown, woolly new growth and pods, with flowering recorded from September to April (VicFlora 2025, Maslin 2012a). It occurs in two disjunct areas where it is extremely localised, the Nunniong Plateau in the Alpine National Park (NP), Vic, and Wadbilliga NP in south-eastern NSW (Figure 1). The morphology of *A. lucasii* varies between locations, with populations in Victoria differing from those in NSW in the size of the gland on the phyllode margin and its distance to the pulvinus. The Extent of Occurrence (EOO) is estimated at 16 km$^2$ based on accepted, post-1970 records from the Victorian Biodiversity Atlas (DELWP 2021). Early maturity is a reproductive trait of *Acacia* species/*lucasii* (Gibson *et al.* 2011) and surveys within 2–3 years post-fire recorded flowering seedlings (see Figure 2).

*Acacia lucasii* is most closely allied to *A. costiniana*, a taxon restricted to the Tinderry Mountains in southern NSW (Figure 1), where it occurs on granitic slopes and sheltered gullies in dry sclerophyll forest and heath communities at an elevation of approximately 1200 m (Maslin 2012b). *Acacia costiniana* is distinguished from *A. lucasii* by its arching branches and green phyllodes, the latter being generally smaller, distinctly undulate and having a sparse covering of shorter, straighter appressed hairs (Figure 2, Maslin 2012b). *Acacia* aff.

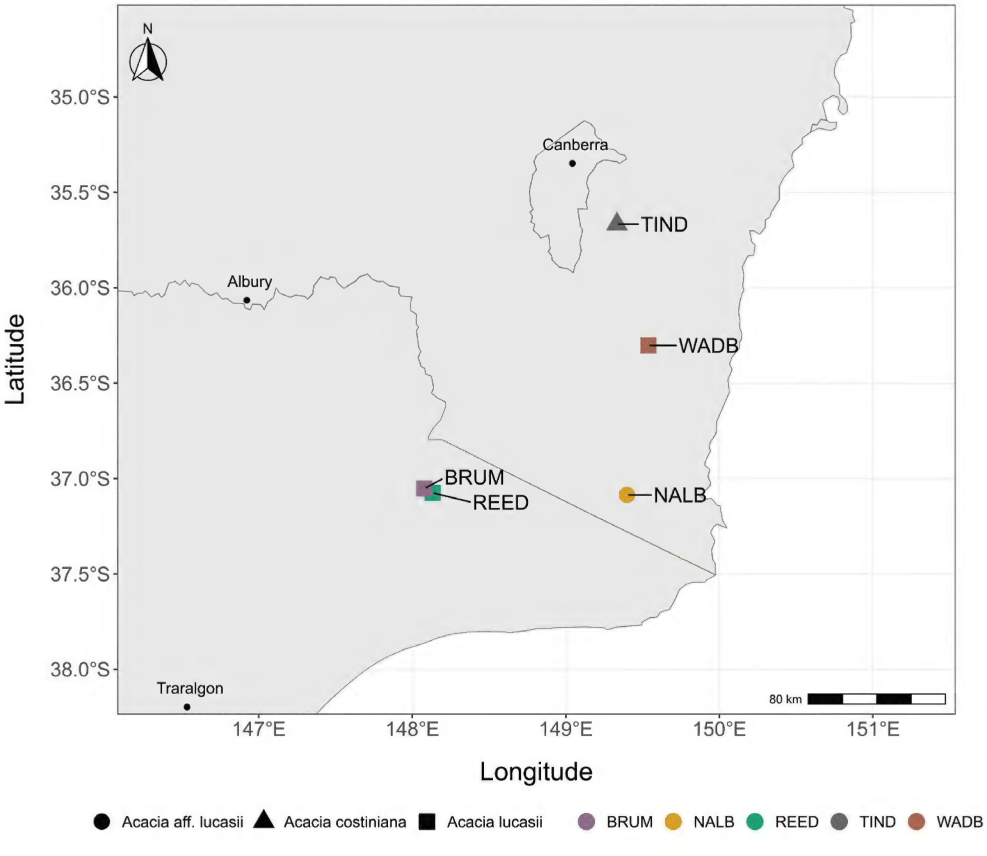

**Figure 1. Map of *Acacia* species and populations sampled for this study.**

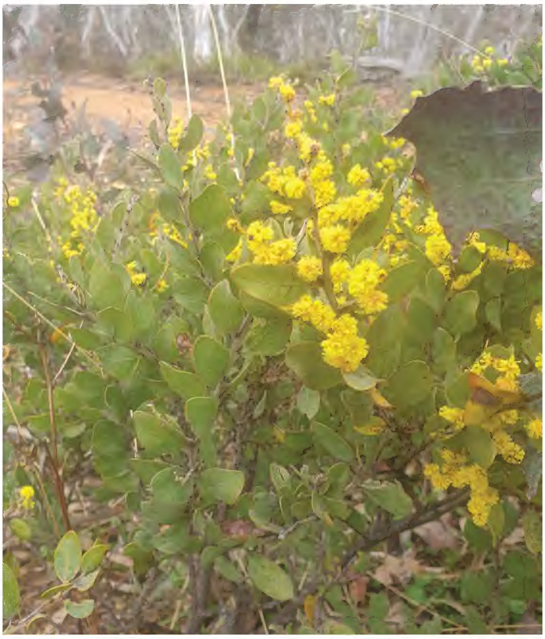

**Figure 2. Photograph of *Acacia lucasii* (image courtesy of authors).**

*lucasii* is found at elevations of approximately 1000 m on the Nalbaugh Plateau in South East Forests NP in southern NSW and differs from *A. lucasii* in having pendulous or sub-pendulous branchlets, usually more undulate phyllodes clothed with non-crisped hairs, glabrous corollas and pods with sparser non-crisped hairs.

## Sampling

Field surveys to locate populations of *Acacia lucasii* were made in December 2021, September 2022, and March 2023. Targeted locations were based on specimen records from the National Herbarium of Victoria and National Herbarium of NSW. All sampled populations were confirmed as burnt in the Black Summer megafire and standing plants were juveniles that appeared to have recruited post-fire. Samples of *A. lucasii* were collected from the two sub-populations on the Nunniong Plateau in Alpine NP, Brumby Point (BRUM) and Reedy Track (REED) which are located on separate ridge lines (Table 1, Figure 1). Both populations were approximately 100 individuals in size. Samples were also collected from a single sub-population of approximately 100 individuals covering 300 m on Wadbilliga Road in Wadbilliga NP (WADB; Table 1). Although the overall extent of *A. lucasii* in Wadbilliga NP is larger compared to the Nunniong Plateau populations, sampling was restricted to sub-population WADB because the additional 3–4 subpopulations were inaccessible due to bushfire, then flood damage at the time of sampling. This included sub-populations located along the northern end of Bumberry Creek Firetrail and at Conways Gap. For comparison, *A. costiniana* was sampled from adult plants in Tinderry Nature Reserve (TIND) which was unburnt in the Black Summer fires, and seedlings of the informal taxon *A.* aff. *lucasii* were sampled from the Nalbaugh Plateau in South East Forests NP (NALB) where all adults had been consumed by fire (Figure 1, Table 1). At each location, five leaves were collected from five individuals spaced at minimum intervals of 5–10 m, placed in labelled envelopes and dried on silica gel.

## DNA extraction, amplification and sequencing

Approximately 15 mg from each of the 25 silica-dried leaf samples (plus 3 technical repeats) were subsequently sent to the commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq™ analysis, a reduced representation sequencing method proven for use in *Acacia* (Kilian *et al.* 2012; van der Merwe *et al.* 2021). DNA was extracted at DArT™ using the NucleoMag Plant kit (Machery Nagel, Germany) on a Tecan 100 platform following the manufacturers protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and

**Table 1. Location and sampling details for the *Acacia* samples used in this study including taxon details.**

| Taxon | Site | Latitude | Longitude | Location |
|---|---|---|---|---|
| *Acacia lucasii* | BRUM | -37.0519 | 148.0789 | Brumby Point Alpine NP, Victoria |
| | REED | -37.0747 | 148.1319 | Reedy Track Alpine NP, Victoria |
| | WADB | -36.3023 | 149.5385 | Wadbilliga Rd Wadbilliga NP, New South Wales |
| *Acacia costiniana* | TIND | -35.6667 | 149.3333 | Tinderry NR, New South Wales |
| *Acacia* aff. *lucasii* | NALB | -37.0858 | 149.3992 | Nalbaugh Plateau SE Forests NP, New South Wales |

MseI and uniquely barcoded adaptors. Following PCR and quantification, samples were standardised and pooled for sequencing in a single lane of an Illumina HiSeq2500. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT's proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DArTsoft14).

## Data analysis

DArTSeq DNA sequence data contained 27 samples and 30,827 loci, with one failed sample (ALUC_2342 from BRUM). All analyses were conducted in R (R Core Team 2019) version 4.4.0 unless otherwise specified. Initial screening for clones was made by separating the dataset into broad taxonomic groups and using Euclidean genetic distance calculations using the R package dartR (Gruber *et al.* 2019). A threshold to select clonal samples was calculated as the average value between repeat pairs plus/minus three standard deviations of the repeat pair values. Any sample pairs above these values in the genetic distance measure were considered clonal samples. A kinship analysis using PLINK method of moment (MoM) for the Identity-By-Descent analysis in SNPRelate (snpgdsIBDMoM; Zheng *et al.* 2012) was used to verify monoclonal individuals. Two datasets were used for data analyses as outlined below.

Dataset 1 comprising all samples of *A. lucasii, A. costiniana* and A. *aff. lucasii,* was used to investigate genetic diversity and differentiation within and among the taxa. Data were filtered using the R packages dartR (Gruber *et al.* 2018), Poppr (Kamvar *et al.* 2014, 2015) and SNPRelate (Zheng *et al.* 2012) in R (R Core Team 2019). To enhance data quality yet retain SNPs across broader phylogenetic groups, the data were filtered for secondaries, read depth (5–150), a reproducibility threshold of 0.95, loci call rate of 0.65, individual call rate of 0.3, minor allele frequency (MAF) set at 8% and for Hamming distance to remove possible paralogues and monomorphic loci which were removed using the R package dartR (Gruber *et al.* 2019). One *A. costiniana* individual from TIND was removed in these steps to give the final Dataset 1 comprised of 2,722 SNPs across 23 individuals.

A phylogenetic network was constructed with the NeighborNet method using a Euclidian Distance matrix, which is not as sensitive to missing data as other options, in the R package (ver. 0.1.0, see http://rdrr.io/github/ IVS-UZH/RSplitsTree/) and visualised in R using ggplot2 (ver. 3.4.2; Wickham 2016) and tanggle (ver. 1.0.0, see http://rdrr.io/github/KlausVigo/tanggle/). Unlike bifurcating trees, phylogenetic networks can capture complex evolutionary histories (e.g., reticulation events) and thus may be more informative when assessing relationships among closely related species (Huson and Bryant 2006).

To visualise the major axes of variation between clusters of individuals, populations and species, principal component analysis (PCA) was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011) in R (R Core Team 2019). Pairwise population differentiation ($F_{ST}$) and isolation by distance (IBD) was assessed using dartR (Gruber *et al.* 2018), analysis of molecular variance (AMOVA) was tested using Poppr (Kamvar *et al.* 2014, 2015) and geographic distance between populations was calculated using GenAlEx 6.503 (Peakall & Smouse 2006, 2012).

A Bayesian analysis implemented in STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000) was used to identify K genetic clusters in the dataset without any information regarding taxon assignment or geographic proximity. The upper K limit was set to six on the assumption that there could be three potential species but five potential genetic groupings based on population disjunctions. Ten independent runs were undertaken for each K value with a burn-in of 100,000 and 200,000 MCMC iterations. The R package pophelper (Francis 2017) and ggplot2 (Wickham 2016) were used to visualise results and select the most probable K based on the ΔK metric (Evanno *et al.* 2005).

Dataset 2 was used to examine levels of genetic diversity in populations of *Acacia lucasii* only. It comprised samples from the three *A. lucasii* populations and contained 17,731 SNPs across 14 individuals prior to filtering. This second dataset was filtered as described above with the loci call rate set at 0.60, individual call rate of 0.35 and MAF set to 7% to produce the final *A. lucasii* Dataset 2 comprised of 1,205 SNPs across the 14 individuals. For both datasets, global heterozygosity statistics were calculated and expected and observed heterozygosity, private alleles and inbreeding coefficients were assessed using the adegenet (Jombart & Ahmed 2011), hierfstat (Goudet & Jombart 2020) and

**Table 2. Pairwise geographic distance in km (top triangle) and pairwise $F_{ST}$ values between each population sampled in this study (bottom triangle), calculated for Dataset 1 (23 individuals, 2,722 SNPs).**

| **Taxon** | | ***A. lucasii*** | | | ***A. costiniana*** | ***A.* aff. *lucasii*** |
|---|---|---|---|---|---|---|
| | **Site** | BRUM | REED | WADB | TIND | NALB |
| *A. lucasii* | BRUM | - | 6 | 177 | 192 | 147 |
| | REED | 0.151 | - | 173 | 189 | 141 |
| | WADB | 0.377 | 0.367 | - | 65 | 77 |
| *A. costiniana* | TIND | 0.444 | 0.461 | 0.411 | - | 136 |
| *A.* aff. *lucasii* | NALB | 0.585 | 0.546 | 0.427 | 0.567 | - |

Poppr (Kamvar *et al.* 2014, 2015) R packages. IBD and AMOVA were performed as detailed above.

# Results

## Species level genetic differentiation and structure

Dataset 1 had an overall nuclear genetic diversity of 0.039, and overall genetic differentiation ($F_{STp}$) value of 0.438. The Victorian populations of *A. lucasii* (BRUM, REED) are the most closely located populations, separated by only 6 km but separated from the NSW *A. lucasii* population WADB by 173 and 177 km respectively (Table 2). Pairwise population differentiation ($F_{ST}$) between the *A. lucasii* populations were 0.151 between BRUM and REED, 0.367 between REED and WADB and 0.377 between BRUM and WADB (Table 2). Pairwise $F_{ST}$ values were higher between the *A. lucasii* and *A. costiniana* populations, ranging from 0.411-0.461. The *A.* aff. *lucasii* population NALB is the most geographically isolated population (77–147 km from any population) and had the highest pairwise $F_{ST}$ values with other populations, ranging from 0.427 to 0.585 (Table 2).

The NeighborNet phylogenetic network of Dataset 1 displays four groupings of individuals, and the differentiation between sampling locations and species

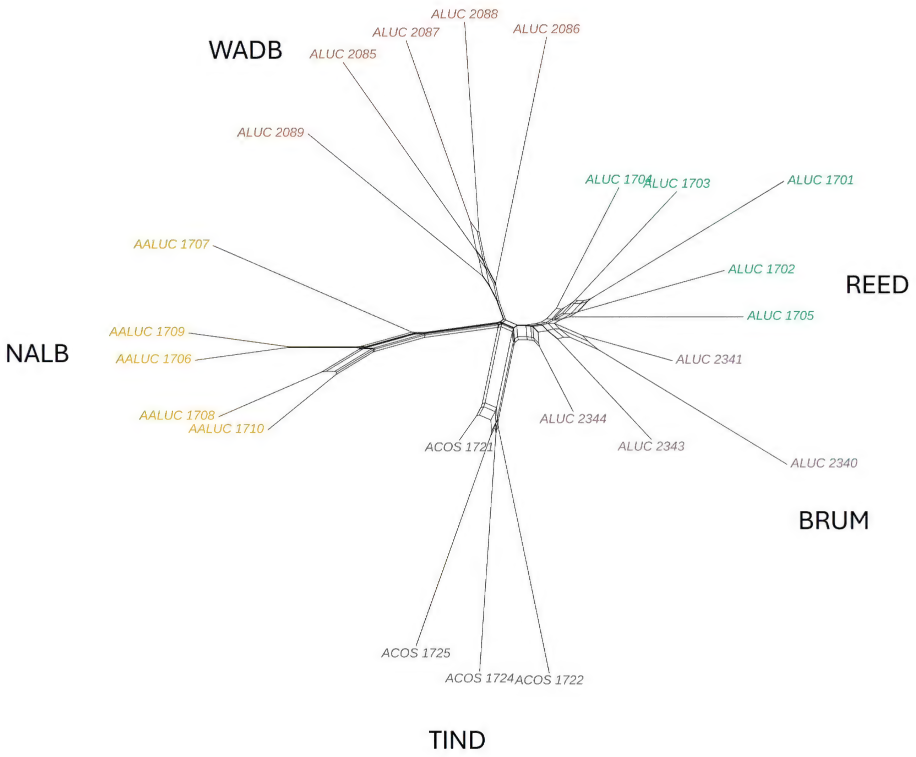
WADB
ALUC 2088
ALUC 2087
ALUC 2086
ALUC 2085
ALUC 2089
ALUC 1704
ALUC 1703
ALUC 1701
AALUC 1707
ALUC 1702
REED
ALUC 1705
AALUC 1709
NALB
AALUC 1706
ALUC 2341
AALUC 1708
AALUC 1710
ALUC 2344
ACOS 1721
ALUC 2343
ALUC 2340
BRUM
ACOS 1725
ACOS 1724
ACOS 1722
TIND

**Figure 3. NeighborNet phylogenetic network based on a Euclidian Distance matrix, among the 23 samples of Dataset 1, with sample name coloured and labelled by location on outside of network. *Acacia lucasii* (BRUM, REED and WADB), *A. costiniana* (TIND), *A.* aff. *lucasii* (NALB).**

**Table 3. The results of AMOVA on for Dataset 1 of 23 individuals, 2,722 SNPs across the three taxa, and Dataset 2 of 14 individuals, 1,205 SNPs across the three *Acacia lucasii* populations. All results presented were statistically significant (p<0.001) at the 95% confidence level.**

| | Dataset 1 | | | Dataset 2 | | |
|---|---|---|---|---|---|---|
| | **Df** | **Sum of Squares** | **Percentage of variation** | **Df** | **Sum of Squares** | **Percentage of variation** |
| Between populations | 4 | 1686.7 | 41.4 | 2 | 439.4 | 29.5 |
| Between samples within populations | 18 | 1548.7 | 38.8 | 11 | 735.5 | 49.2 |
| Within samples | 23 | 401.4 | 19.8 | 14 | 167 | 21.4 |
| Total | 45 | 3636.8 | 100 | 27 | 1341.9 | 100 |

(Figure 3). *Acacia costiniana* and *A.* aff. *lucasii* had the longest splits indicative of greater genetic distance compared to the distance between populations of *A. lucasii*. The Victorian populations of *A. lucasii*, REED and BRUM, share a branch, with separation of the populations towards the tip. The NSW population of *A. lucasii*, WADB, clusters separately from the Victorian populations. The individuals from the *A. costiniana* population from TIND cluster together on a longer branch than the *A. lucasii* populations which could reflect the taxonomic divergence of this species. Finally, the *A.* aff. *lucasii* population also clusters on a long branch away from the other taxa, consistent both with this taxon's isolation and possible taxonomic divergence based on differences in morphology.

The principal component analysis of Dataset 1 (Figure 4) displays the taxonomic separation in the dataset with all samples of *A. lucasii* separated from *A.* aff. *lucasii* along the first axis and separation of *A. costiniana*, across the second axis. The first axis explains 15.47% of the variation in the dataset and despite the geographic distance between the three populations of *A. lucasii*, these cluster together in the bottom right side of the plot and are clearly separated from *A.* aff. *lucasii* (Figure 4a). The second axis explains 13.64% of the variation in the dataset which separates the population of *A. costiniana* from the three *A. lucasii* populations. The New South Wales population of *A. lucasii*, WADB is separated from the Victorian populations BRUM and REED across the third axis (11.48% of variation) reflecting the geographic distance between populations of this taxon (Figure 4b).

The Evanno ΔK method identified K = 5 clusters in the STRUCTURE analysis, however there was also a peak at K = 3 (see Supplementary Material 1, available online), thus both K = 3 and K = 5 charts are presented in Figure 5. At K = 3 the Victorian *A. lucasii* populations, REED and BRUM, belong to the same cluster while the NSW population of this taxon, WADB, shows admixture between the Victorian *A. lucasii* cluster and a second cluster (Figure 5b). The Nalbaugh Plateau population of *A.* aff. *lucasii* is primarily composed of the second cluster and shows admixture with a third cluster which is found primarily in *A. constiniana*. At K = 5, each cluster primarily represents one population (Figure 5a). Some admixture with the genetic cluster from the adjacent REED population is present in all individuals from the *A. lucasii* population BRUM. One individual from NALB has small amounts of admixture from the BRUM and the WADB clusters.

The AMOVA for Dataset 1 showed similar levels of genetic variation between the five populations (41.4%) and between the samples within the populations (38.8%) while only 19.8% was explained between the samples (Table 3, Dataset 1). There was a significant positive correlation between genetic distance and geographic distance ($r = 0.592$, $p = 0.042$).

## Genetic diversity in *Acacia lucasii*

Nuclear genetic diversity for *A. lucasii* (Dataset 2) across all populations was found to be higher than the overall Dataset 1 at 0.054, with a corrected species genetic differentiation ($F_{STp}$) of 0.319. Genetic diversity values for the *A. lucasii* populations are shown in Table 4. Allelic richness ($A_R$) was highest in the NSW population WADB (1.30) and lowest in BRUM (1.24). The number of private alleles in *A. lucasii* populations ranged from a low of 166 (BRUM) to a high of 398 (WADB), suggesting that gene flow is restricted as detected in the previous genetic differentiation results. For all sampled populations, observed heterozygosity ($H_O$) was consistently lower than expected heterozygosity ($H_E$), resulting in

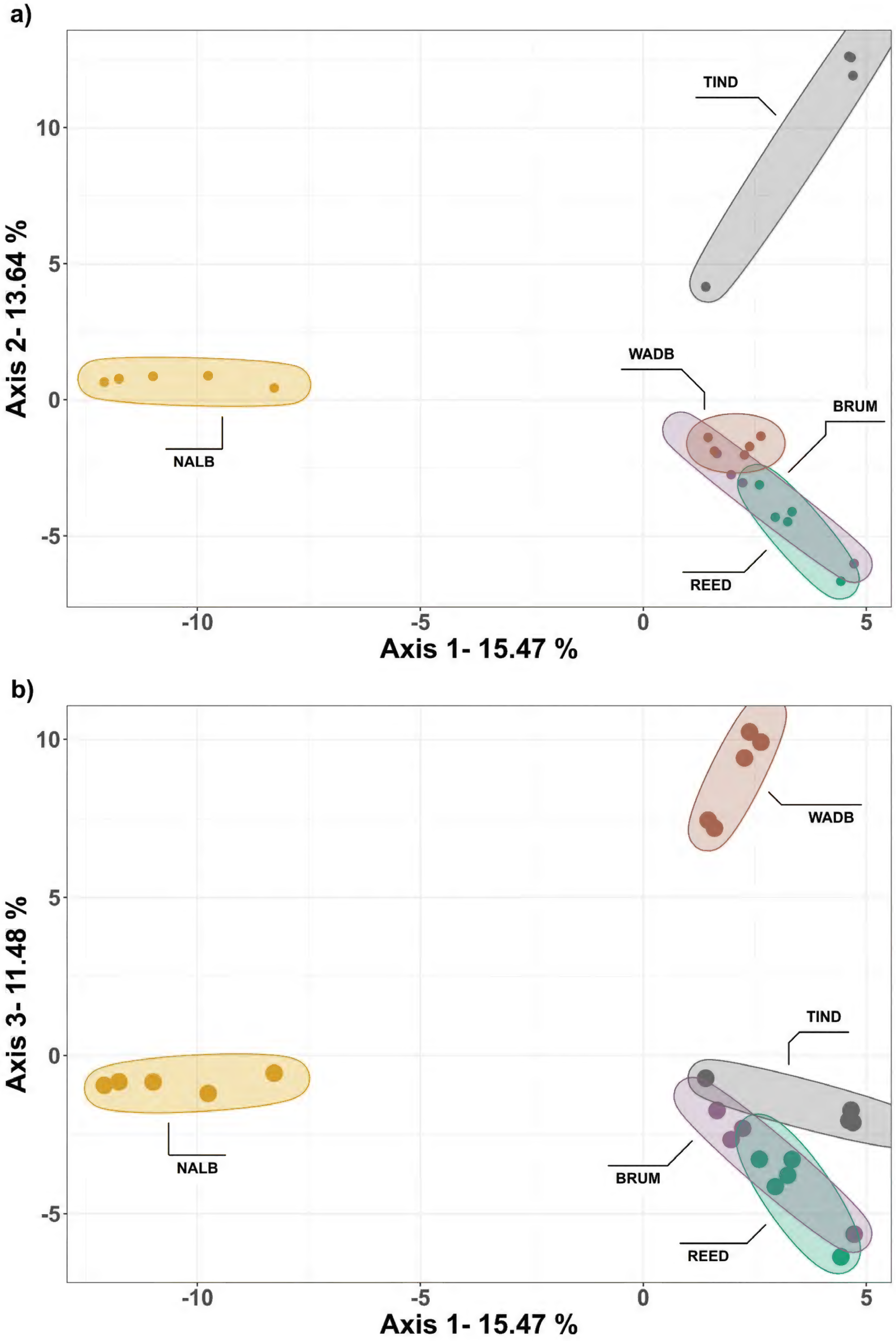

**Figure 4. Plots of the principal component analysis of Dataset 1 (23 individuals, 2,722 SNPs), (a) the first and second axis, (b) the first and third axis. Individual samples are coloured by their population of origin with shapes representing taxa, *A. lucasii* = diamonds, *A. costiniana* = triangles, *A.* aff. *lucasii* = circles.**

**Table 4. Genetic diversity characteristics of the *Acacia lucasii* populations based on Dataset 2 with 1,205 SNPs and 14 individuals. Standard error is provided in brackets.**

|  | No. individuals | No. Private alleles | Mean allelic richness ($A_R$) | Expected heterozygosity ($H_E$) | Observed heterozygosity ($H_O$) | Inbreeding coefficient ($F_{IS}$) |
|---|---|---|---|---|---|---|
| BRUM | 4 | 166 | 1.24 (0.01) | 0.147 (0.005) | 0.023 (0.002) | 0.843 |
| REED | 5 | 233 | 1.29 (0.01) | 0.179 (0.004) | 0.036 (0.002) | 0.798 |
| WADB | 5 | 398 | 1.30 (0.01) | 0.192 (0.004) | 0.104 (0.003) | 0.461 |

inbreeding coefficients ($F_{IS}$) for *A. lucasii* that ranged from 0.461 (WADB) to 0.843 and 0.798 for the Victorian populations REED and BRUM, respectively (Table 4).

In contrast to Dataset 1, the AMOVA for Dataset 2 found highest levels of genetic variation between samples within the populations (49.2%), compared to that between populations (29.5%) and within all samples (21.4%, Table 3). A strong but not significant correlation between genetic distance and geographic distance (IBD) was detected ($r = 0.988$, $p = 0.166$).

## Discussion

This study examined genomic variation in populations of the disjunct and threatened *Acacia lucasii* and to two regionally and closely related congeners, *A. costiniana* and *A.* aff. *lucasii*. The geographic ranges of the taxa do not overlap, and each occurs in highly isolated populations. Our results suggest that *A. lucasii* comprises two geographically disjunct genetic lineages that have a close affinity to two lineages represented by *A. costiniana* and *A.* aff. *lucasii*. The genetic evidence

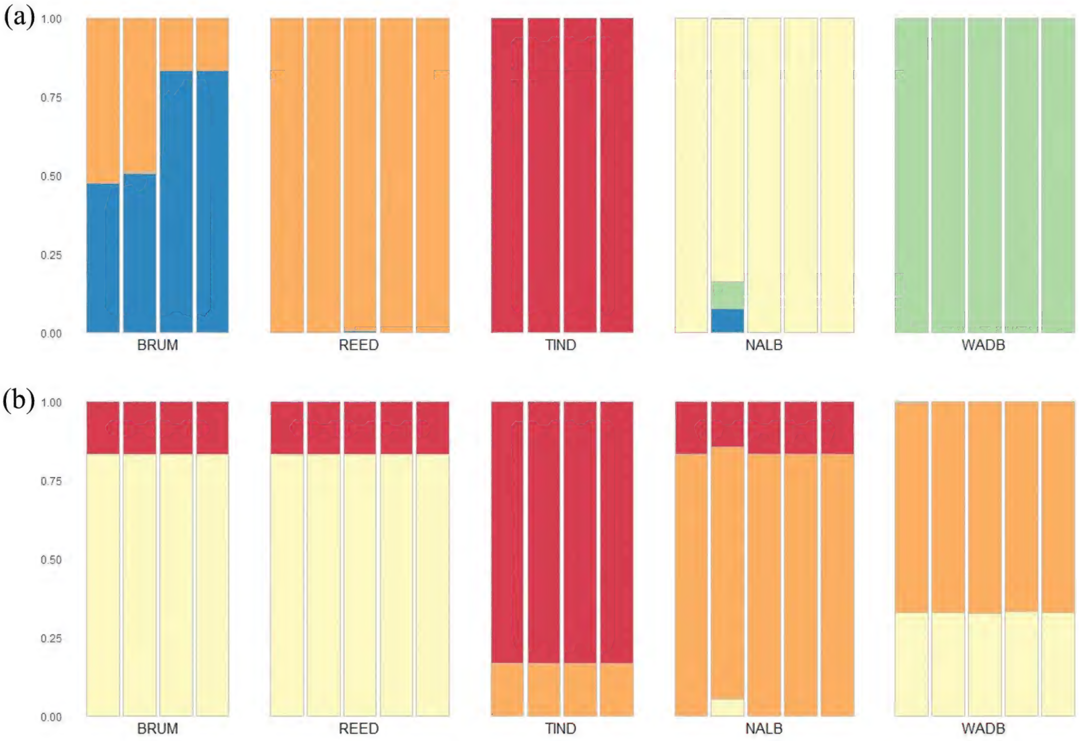

**Figure 5. The results of the STRUCTURE analysis on Dataset 1 of 23 individuals and 2,722 SNPs with (a) the STUCTURE results displaying bar chart of individual Q-membership proportions to each of K = 5 clusters, with individuals clustered by population and populations labelled below (b) the STRUCTURE results displaying bar chart of individual Q-membership proportions to each of K = 3 clusters, with individuals clustered by population and populations labelled below.**

provides support for delimitation of a minimum of three taxa (taxon 1 -TIND (*A. costiniana*); taxon 2 – BRUM, REED, WADB (*A. lucasii*); and taxon 3 – NALB (*A.* aff. *lucasii*)) or four taxa (taxon 1 -TIND (*A. costiniana*); taxon 2 -WADB (*A. lucasii*); taxon 3 -BRUM, REED (*A. lucasii*); and taxon 4 -NALB (*A.* aff. l*ucasii*)) subject to further examination of morphological differences. The inconsistency in conservation status across different jurisdictions for *A. lucasii* (Critically Endangered in Victoria but no conservation listing in NSW), and the likelihood of an unrecognised, distinct entity, *A.* aff. *lucasii*, means that conservation resources are not distributed evenly, despite the rarity of each genetic lineage. These results exemplify the argument that genomic analysis is essential for the taxonomic assessment of morphologically similar taxa prior to conservation listing (Cascini *et al.* 2025). The inclusion of an evolutionary perspective is a goal often cited in conservation management (Moritz & Potter 2013) but can be difficult to implement (Cook & Sgrò 2019). Deciding where to draw the line between divergence that reflects differentiation between disjunct intraspecies populations and the divergence that typifies interspecific differentiation is complex in cases such as this (Stankowski & Ravinet 2021), particularly when data sets are small and sampling may not have included the full known distribution of all taxa.

Effective conservation of rare species such as *A. lucasii* can be complicated by difficulties distinguishing the focal taxon from genetically similar lineages. The presence of cryptic genetic lineages may require management strategies that account for broader genetic relationships to ensure that conservation actions reflect underlying evolutionary patterns and preserve genetic diversity more comprehensively (Ahrens *et al.* 2017). We propose that results of this study tell a narrative that the extant populations of *A. lucasii*, *A. costiniana* and *A.* aff. *lucasii* are remnants of a previously more widespread precursor, whose range has expanded or contracted during historic climatic oscillations and complex geological events, resulting in contemporary geographic separation and divergence (Byrne 2008, Bryant & Krosch 2016). We found low levels of diversity ($A_R$, $H_E$, $H_O$) in each *A. lucasii* population, which fall within the range found in the critically endangered but more populous *A. purpureapetala* (van der Merwe *et al.* 2021) but are lower than those found in other studies of endangered *Acacia* species (Blyth *et al.* 2020, Rossetto *et al.* 2020). Low heterozygosity levels are considered detrimental for long term survival because adaptive capacity and fitness are likely to be compromised (Reed & Frankham 2003, Leimu *et al.* 2006). The results presented here therefore confirm a level of genetic risk for *A. lucasii*. The low heterozygosity at sites suggests biparental inbreeding in small, genetically depauperate populations leading to genetic bottlenecking, genetic drift and the fixation of alleles (Frankham *et al.* 2002). $F_{ST}$ values indicate that gene flow is unlikely to have occurred in any recent timeframe except between the more geographically close Victorian populations of *A. lucasii* (REED and BRUM). Realistically, even the 6 km distance between these populations may be beyond ant- or bird- seed dispersal distance, at the limits of invertebrate or bird pollination distance, and long distance abiotic dispersal events could be rare (Whitney 2002, Stone *et al.* 2003, Gibson *et al.* 2011). Thus, gene flow between these populations may be associated with either undiscovered populations in inaccessible areas or represent a bygone time where the species' distribution may have been more contiguous in the landscape. High levels of genetic divergence ($F_{ST}$) can follow isolation, where small populations drift quickly, leading to fixed differences between populations. The apparent introgression of genetic clusters identified in the STRUCTURE analysis for $K = 3$ is also consistent with a scenario of retained sequence similarity from a more common precursor that underwent range contraction and fragmentation in concert with historical climate change in southeastern Australia. Fragmentation, both natural as landscape complexities have evolved, or human mediated, could have enhanced genetic drift now evident as the separate genetic lineages identified at $K = 5$.

## Conservation implications

While the loss of populations and reduction in population sizes of *Acacia lucasii* or its ancestors are not necessarily recent or human-induced, it does not lessen the importance of minimising within-taxon loss of diversity, and concomitant adaptive capacity, or the risk of incremental species loss which may compromise the functional integrity of ecological communities

(Halley & Pimm 2023). The inconsistency in conservation status across different jurisdictions for *A. lucasii,* and the likelihood of an unrecognised but distinct entity, *A.* aff. *lucasii,* means that conservation resources are not distributed evenly despite the rarity of each genetic lineage. This supports the argument for genomic analysis to assist in the taxonomic assessment of morphologically similar taxa prior to conservation listing (Cascini *et al.* 2025). To reconcile the current taxonomy, sampling of the additional sub-populations of *A. lucasii* in Wadbilliga NP and the inclusion of an additional sub-population of *A. costiniana* from the northern Tinderry Ranges would be needed to confidently identify where populations lie on the speciation continuum. More intensive sampling across populations of all taxa would assess fine scale structuring, spatial autocorrelation and breeding patterns, which could be useful for seed collection protocols.

A precautionary approach may be to consider the conservation of each population as individual conservation units, or applying units to conserve the Nalbaugh entity *Acacia* aff. *lucasii* and the Victorian populations of *A. lucasii* separately from NSW populations of *A. lucasii*, as these represent genetically defined lineages (Mace 2004). This approach would be similar to that used for a group of rare, leafless *Bossiaea* species also from southeastern Australia where genomic analysis has enabled a revision of previous taxonomy based on morphology and led to changes in the approach to the conservation of the group as a whole (McMaster *et al.* 2024). Irrespective of taxonomic rank, the use of mixed germplasm sources for genetic rescue between conservation units is not recommended as it may be detrimental given the distinct lineages, geographic disjunctions and high differentiation. A manipulated exchange of germplasm by either broadcasting seed or planting seedlings from the opposite, closely situated Victorian populations BRUM and REED could boost genetic diversity and reduce the high degree of inbreeding within each of these populations and reduce the risk of extinction.

Although only *Acacia lucasii* and *A.* aff. *lucasii* were burnt by the Black Summer bushfires, all three study taxa are potentially threatened by inappropriate fire regimes and climate change because of their small geographic distribution and EOO, and inability to disperse or re-colonise from sources other than local. These obligate seeders rely on the establishment of a soil seed bank between fires and subsequent population re-establishment through germinating seed post-fire for their persistence in the landscape. Fire suppression such as management intervention to protect assets may inhibit regeneration due to competition from co-occurring species, whilst an increase in fire frequency and intensity may deplete the taxon's seed bank and thus its potential for recruitment post-fire (DELWP 2021). Early maturity is a reproductive trait of wattles (Gibson *et al.* 2011) and seedlings of *A. lucasii* were observed in flower at WADB within two years of the Black Summer fires, thus appearing to be responding positively. However, all sampled plants were juveniles that appeared to be new recruits post-fire from the soil seed bank. Even with several years of reproduction, the soil seedbank, at best, will only encompass the genetic diversity present in the standing stock. Assisted gene flow via population augmentation with non-local seed is feasible but potential genetic risks would need prior careful consideration. The occurrence in national parks affords some level of protection from external threats, however herbivory from animals, misapplication of fire intervals for prescribed burns, or repeated bushfires prior to the re-establishment of seedbanks remain as threats to the survival of each population in this study (Auld *et al.* 2022).

The small number of populations makes *ex situ* germplasm collections a feasible proposition to provide a rapid insurance population for *Acacia lucasii* and its close congeners. Given the apparent early reproductive maturity post-fire, *ex situ* seed banking is likely to be a cost-effective strategy to conserve genetic diversity and can be combined with population monitoring to understand post-fire population recovery. Multiple conservation seed collections from the NSW distribution of *A. lucasii* and of *A. costiniana* made prior to the Black Summer fires are held at the Australian Plantbank (Sydney) and National Seedbank (Canberra), and a small seed collection of *A. lucasii* from Brumby Point made in 2025 is held at the Victorian Conservation Seedbank (Melbourne). Additional sampling of seed from Reedy Track and repeated collections across multiple years for both populations of *A. lucasii* in Victoria would ensure that *ex situ* germplasm is as genetically representative as possible. Collections of *A.* aff. *lucasii* would complete the *ex situ* conservation holdings for each lineage of this group. The availability of *ex situ* seed collected and stored as separate maternal lines (Commander *et al.* 2018) allows for the possibility of translocations or population augmentation in the case of future catastrophic events that impact the viability of *in situ* populations.

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Muelleria

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# Seeing the tree through the forest, *Eucalyptus mackintii* (Myrtaceae), how to conserve the hard to ID

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## Introduction

Genetic diversity underpins the health and fitness of populations and the ability they have to adapt to future challenges (Booy *et al.* 2000). Genetic diversity is a commonly used criterion-based evaluation of a species' conservation status and is included in the Global Biodiversity Framework (Stephens 2023). While not incorporated into the IUCN Red List risk assessments, other assessment criteria such as population size, population fragmentation and range size are included and are often related to genetic diversity (Schmidt *et al.* 2023). Extent of occurrence and area of occupancy are included in evaluating a species' geographic range size which, when limited, places a species at a high risk of extinction particularly when populations are severely fragmented (IUCN 2012). Range sizes that are large can secure a species against localised threats and reduce the chance of range-wide impacts. Conserving species that have restricted ranges is not easy as they are more vulnerable to threats impacting the whole species. Australia has an increasing risk of extreme weather and fires, raising the chances of a range-wide catastrophic event for species with small ranges (Collins *et al.* 2022; Ellis *et al.* 2022). Loss, or a reduction in size, of populations of range-restricted species can reduce genetic diversity if gene flow between populations is restricted (Ellstrand & Elam 1993; Oostermeijer *et al.* 2003).

Population genetic studies can provide practical information on landscape genetic patterns to assist in the design of collection strategies

## Abstract

*Eucalyptus mackintii* Kottek (Gippsland stringybark) is endemic to East Gippsland, Victoria and is recovering after widespread fires in 2019/2020 severely impacted the species at several locations. This study estimated levels of genetic diversity and connectivity among nine locations across the species' range. With high levels of gene flow across the distribution, on-ground management should focus on maintaining this connectivity and preventing population fragmentation. The greatest challenge to managing this species is accurate identification. It is recommended that sampling is expanded, spatially and temporally, for *ex situ* seed collections so they represent the genetic diversity found in this study and that collections are made by those trained in *Eucalyptus* identification to ensure propagules are collected from the correct species.

***Keywords*:** conservation, DArTseq, genetics, restricted distribution, resprouting

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for *ex situ* germplasm and its use for threatened species conservation. Naturally high gene flow maintains genetic diversity, reduces the risk of inbreeding (Frankham 2015) and reduces the genetic differentiation between populations. If populations are not naturally fragmented and gene flow is high, differences in local adaptation may be minimal (Lenormand 2002; Savolainen *et al.* 2007) but there could still be potential for adaptation resulting from additive genetic variance (Noble *et al.* 2019). An understanding of patterns of genetic diversity as a proxy for local adaptation can highlight populations most susceptible to climate-induced stressors (Meek *et al.* 2023). Historical patterns of genetic connectivity are not necessarily maintained under contemporary land-use pressures. Agriculture, timber harvesting, and changing climates have the potential to disrupt gene flow. This may necessitate targeted conservation efforts to re-establish connectivity and maintain genetic diversity. Where site conditions have changed, conservation decisions regarding the use of germplasm are likely to be a compromise between restoring the presumed historical landscape genetic patterns and providing highly diverse genetic material for restoration under the premise that greater diversity will ensure that some genotypes will be suited to the modified habitat or climatic conditions (Meek *et al.* 2023). However, standing genetic variation in range-restricted species, or some populations therein, may be insufficient to facilitate local adaptation under new conditions (Barrett & Schluter 2008).

Genetic diversity is essential for maintaining adaptive potential which enables populations to persist under changing environmental conditions (Barrett & Schluter 2008). Globally, fire activity is projected to increase, and fires are predicted to become larger and more severe in southeastern Australia (Collins 2020; Ellis *et al.* 2022). Under conditions sub-optimal for seed production or recruitment, asexual reproduction, such as resprouting or root-suckering is considered an adaptive mechanism for persistence (Vallejo-Marín *et al.* 2010). In response to fire, some plants will resprout or root-sucker, thus maintaining pre-fire genetic diversity and alleviating the reliance solely on a seed bank for survival. Asexual reproduction can maintain genetic diversity or slow down its loss, but the production of novel genotypes is reliant on sexual reproduction (Silvertown 2008). Dominant across many vegetation communities in Australia, *Eucalyptus* species are well known across fire prone landscapes. In general, they are split into two fire response groups, post-fire resprouters and seeders (Burrows 2013). While resprouting can maintain extant genomic diversity, repeated fires may reduce the likelihood of resprouting and eventually plants will senesce (Fairman *et al.* 2019) resulting in erosion of standing genetic diversity and adaptive potential.

Given the variable nature of *Eucalyptus* species, it is important to understand the genetic, reproductive and gene flow dynamics on a case-by-case basis to guide appropriate management and seed collection. Gene flow via pollen and seed in *Eucalyptus* generally occurs over relatively short distances. Dispersal of pollen is typically less than 200 m from its source but dispersal over 1 km has been recorded (Ford *et al.* 1979; House 1997; Hingston *et al.* 2004; Griffin *et al.* 2009; Booth 2017). The majority of seed from *Eucalyptus* species falls within a distance equivalent to the height of the tree (Booth 2017), so long distance gene flow is likely to be mediated by pollination (Potts & Wiltshire 1997). Gene flow and genetic structuring in *Eucalyptus* is also variable, with genetic structuring and restricted gene flow found at short distance in *E. camaldulensis* Dehnh. (Butcher *et al.* 2009) but, more commonly, little genetic structuring and high gene flow, as identified in *E. marginata* Donn ex Sm., *E. pauciflora* Sieber ex Spreng. and *E. wandoo* Blakely (Wheeler *et al.* 2003; Byrne *et al.* 2008; Gauli *et al.* 2014).

*Eucalyptus mackintii* Kottek is a rare tree restricted to a small area (Area of Occupancy (AOO) is estimated to be 120 km$^2$) in East Gippsland, Victoria, and due to its small range it is listed as Vulnerable under the Victorian Government Flora and Fauna Guarantee Act 1988 (DEWLP 2021a; DEWLP 2021b). The widespread 2019/2020 Black Summer Fires impacted approximately 75% of its range, in many locations consuming the entire canopy (DEECA 2025). The species is likely to be insect-pollinated and is a post-fire resprouter. The species now appears to be on a trajectory to recovery as existing trees are resprouting across its range. Eucalypt seedlings have also been observed during post-fire surveys but these could not be identified to species level.

Among recommendations for the future management of *Eucalyptus mackintii*, seed collection from multiple sites for the Victorian Conservation Seedbank (VCS) was prioritised (DEWLP 2022). The restricted and sporadic distribution of *E. mackintii* and its occurrence among other morphologically similar eucalypt species make it difficult to discern the population dynamics and connectivity across the species' distribution in the absence of genetic data. The species has not been included in any genetic studies to date. An understanding of the genetic landscape of *E. mackintii* can guide conservation management activities such as the collection of genetically representative seed for the VCS and the sourcing of germplasm for use in restoration. This study assessed the broadscale genetic diversity of *E. mackintii* and identified localised genetic patterns.

## Methods

### Study species

*Eucalyptus mackintii*, commonly known as the Gippsland or blue-crowned stringybark, is a rare tree restricted to an area from northwest of Bairnsdale to northeast of Orbost in East Gippsland, Victoria (Figure 1). Previously regarded as a hybrid with *E. macrorhyncha* F.Muell. ex Benth. as one of its parents, *E. mackintii* was described as a distinct species in 1990 when morphological and chemical analysis found no evidence to support a hybrid origin (Kottek *et al.* 1990). The taxon has been listed as Vulnerable under the Victorian Government *Flora and Fauna Guarantee Act 1988* since May 2021 (DEWLP 2021a). Until the cessation of native forest logging in January 2024, forestry operations were considered the main risk to *E. mackintii* due to its occurrence in mixed species forest subject to timber harvesting (DEWLP 2022). Other hazards such as wildfires may impact the species, and successive disturbances could reduce recruitment and exacerbate population decline. The species is most at risk in lowland coastal plains where it is a minor species, with individuals scattered amongst other eucalypts. The range of *E. mackintii* is predominantly forested, interspersed with localised clearings and historical logging coupes scattered throughout.

Plants of *E. mackintii* grow up to 30 m tall in coarse sandy loams or clay soils in foothills (Figure 2a). Individuals can respond to fire damage by resprouting from epicormic buds (Figure 2b). Herbarium data indicate sporadic flowering and fruiting over a year (Supplementary Table S1, available online). During surveys for this study, little flowering was observed between June and October 2021 whereas flowering trees were common in December 2021 (Figure 2c).

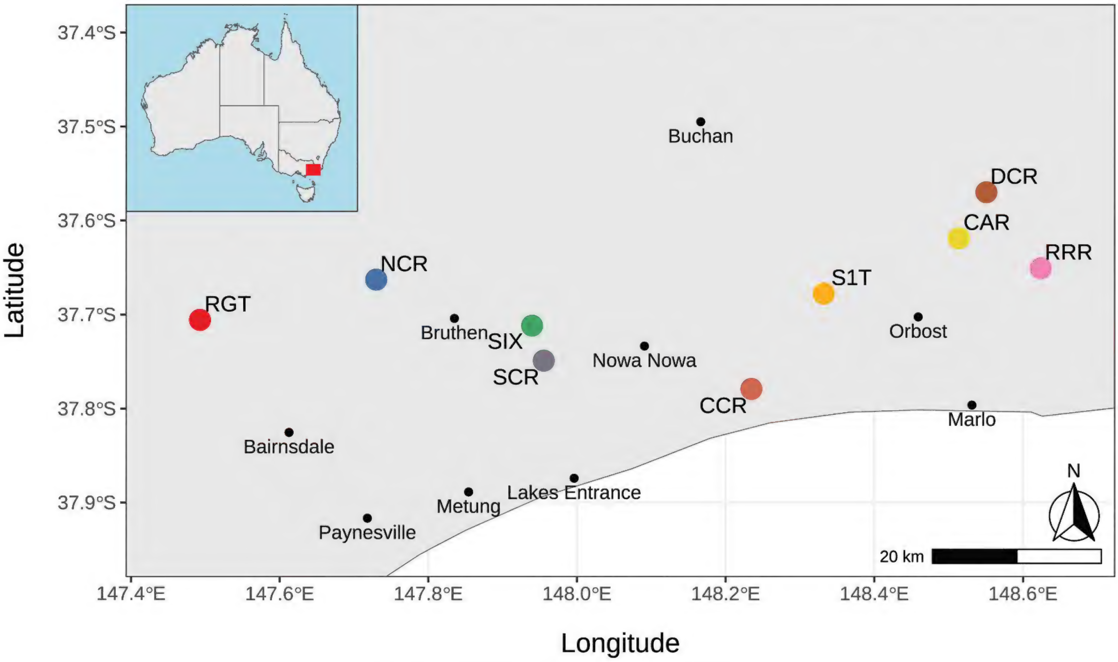

**Figure 1** Locations of sampled populations.

Fruit is a hemispherical to globose capsule (Figure 2d). Rough, orange-brown bark occurs on the trunk and extends to the small branches. Leaves are lanceolate to falcate with an oblique base and slightly glossy and blue-green, giving a distinctive hue to the canopy. Juvenile leaves are oblong to ovate, with an oblique base and distinctive apex (Slee *et al.* 2020).

Adult plants of *Eucalyptus mackintii* can be distinguished from *E. macrorhyncha* by the lack of a distinct beak on the operculum and a fruit disc that is neither broad nor ascending as in *E. macrorhyncha*. Seedlings of *E. mackintii* are indistinguishable from *E. macrorhyncha* (Kottek *et al.* 1990) so it is not possible to determine whether the extensive seed germination seen after fire is from *E. mackintii*, a sympatric eucalypt, or a combination of species.

## Sample collection

Field surveys for this species targeted locations across the species' range from northwest of Bairnsdale to northeast of Orbost based on National Herbarium of Victoria specimens or observation records from Atlas of Living Australia (Australasian Virtual Herbarium 2022). We found this species to grow in patches and only in areas with poor, generally sandy soils but it was commonly observed across the range. The widespread bushfire in 2019/2020 affected a large area of the species' range, so both burnt and unburnt locations were sampled. In burnt locations, juvenile to adult leaves were collected from resprouting trees where fruit was present on the ground to assist in identification.

Leaf samples of *E. mackintii* were collected ~40 m apart (approximately 2 canopy heights) to minimise the

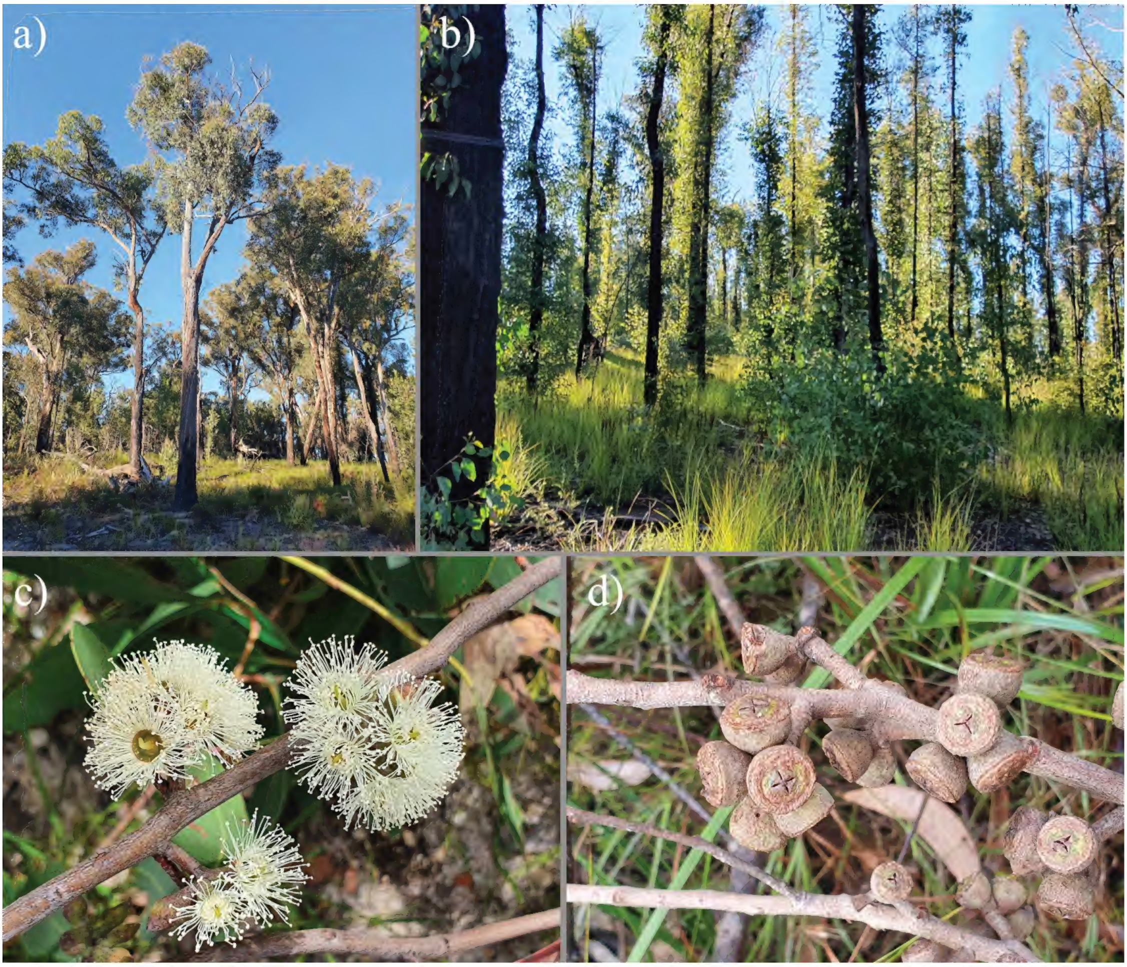

**Figure 2** (a) Habitat of *Eucalyptus mackintii*, (b) re-growth after fire, (c) flowers, and (d) fruit.

**Table 1. Location details for *Eucalyptus mackintii* sample sites.**

| Site | Latitude | Longitude | Location | Burnt |
|---|---|---|---|---|
| **RGT** | -37.706 | 147.493 | Rain Gauge Track | no |
| **NCR** | -37.663 | 147.730 | Nicholson Creek Rd | yes |
| **SIX** | -37.712 | 147.940 | Six Mile Track | no |
| **SCR** | -37.749 | 147.955 | Siberia Crossing Rd | no |
| **CCR** | -37.779 | 148.235 | Circle Rd | no |
| **S1T** | -37.678 | 148.332 | Stoney #1 Track | no |
| **CAR** | -37.619 | 148.514 | Caulson Rd | yes |
| **DCR** | -37.570 | 148.551 | Dynamite Creek Rd | yes |
| **RRR** | -37.649 | 148.621 | Rocky River Rd | no |

chances of sampling siblings as most eucalypt seeds fall within the distance of the canopy height of parent trees (Booth 2017). Samples were made from 4 to 6 individuals at 12 populations. Preliminary results indicated that collections at three populations were likely to be a mix of species, so those sites were removed from the dataset prior to analysis leaving nine populations representing the species across its geographic range (Table 1, Figure 2). This highlights the challenges in management of this species; identification can be difficult, particularly after fires when diagnostic characters can be challenging if not impossible to find.

## DNA extraction, amplification and sequencing

Approximately 15 mg from each of the 65 silica-dried leaf samples (including four technical repeats) were subsequently sent to the commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq$^{TM}$ analysis, a reduced representation sequencing method proven for use in eucalypts (Sansaloni *et al.* 2010; Kilian *et al.* 2012; Cruz *et al.* 2013). DNA was extracted at DArT using the Nucleo Mag Plant C-Bead kit (Machery Nagel, Germany), on a Tecan 100 platform following the manufacturers protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and HpaII and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina HiSeq2500. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT's proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DarTsoft14).

## Data analysis

The results from DArTseq contained 64 samples and 54,065 loci. DNA sequence data received from DArTseq analysis were filtered using the R packages dartRverse v.0.51 (Gruber *et al.* 2018; Mijangos *et al.* 2022) and Poppr (Kamvar *et al.* 2014, 2015) in R v.4.3.3 (R Core Team 2024). The data were filtered to a locus call rate of 0.9 and individual call rate of 0.9, a reproducibility score of 0.95, a Hardy-Weinberg equilibrium with a 5% level of significance, a minor allele count of 2, removal of monomorphic loci, and finally filtered on Hamming distance with a threshold of 0.2 to remove potential paralogues. After filtering, as outlined above, including removal of technical repeats and populations with misidentified individuals, the SNP data set contained 6471 loci and 44 individuals across nine populations.

To identify genetic clusters of individuals and visualise the major axes of variation between clusters, principal component analysis (PCA) was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011) in R (R Core Team 2024). Expected and observed heterozygosity, inbreeding coefficients and pairwise population differentiation ($F_{ST}$) based on the estimator from Weir & Cockerham (1984), using 1000 bootstraps to estimate probability of $F_{ST}$ values to be different from zero, were assessed using the dartRverse (Gruber *et al.* 2018; Mijangos *et al.* 2022), adegenet (Jombart & Ahmed 2011), hierfstat (Goudet & Jombart 2020) and Poppr (Kamvar *et al.* 2014, 2015) packages in R. Overall species genetic differentiation ($F_{STp}$), corrected for sampling error, was calculated using hierfstat (Goudet & Jombart 2020). Analysis of molecular variance (AMOVA) was tested using the Poppr (Kamvar *et al.* 2014, 2015) package in R. Isolation by distance (IBD) and fixed difference analyses were assessed using R package dartRverse (Gruber *et al.* 2018; Mijangos *et al.* 2022). Population genetic structure was explored using Structure v.2.3.4 (Pritchard *et al.* 2000) using K-values from 2 to 11. Ten independent runs were undertaken for each K value with a burnin of 100,000 and 200,000 MCMC iterations. The R packages pophelper (Francis 2017) and ggplot2 (Wickham 2016) were used to visualise results and select the most probable K based on the ΔK metric (Evanno *et al.* 2005). The Evanno method

**Table 2. Genetic diversity characteristics of the populations, standard error in brackets.**

| Site code | Number of individuals | Expected heterozygosity | Observed heterozygosity | Inbreeding coefficient |
|---|---|---|---|---|
| **RGT** | 5 | 0.156 (0.002) | 0.142 (0.003) | 0.148 (0.005) |
| **NCR** | 5 | 0.159 (0.002) | 0.144 (0.003) | 0.155 (0.005) |
| **SIX** | 5 | 0.162 (0.002) | 0.145 (0.002) | 0.162 (0.005) |
| **SCR** | 5 | 0.156 (0.002) | 0.142 (0.002) | 0.151 (0.005) |
| **CCR** | 6 | 0.160 (0.002) | 0.144 (0.002) | 0.148 (0.005) |
| **S1T** | 4 | 0.156 (0.002) | 0.144 (0.003) | 0.160 (0.005) |
| **CAR** | 4 | 0.161 (0.002) | 0.145 (0.003) | 0.173 (0.005) |
| **DCR** | 5 | 0.166 (0.002) | 0.145 (0.002) | 0.174 (0.005) |
| **RRR** | 5 | 0.163 (0.002) | 0.144 (0.002) | 0.170 (0.005) |

can underestimate the optimal value of K and as such we present results for all K values where a peak was identified (Janes *et al.* 2017).

## Results

The analysis of *Eucalyptus mackintii* found overall observed nuclear genetic diversity across all populations to be 0.189. Individual population genetic diversity values are shown in Table 2. The observed heterozygosity values were consistent across populations ranging from 0.142 (RGT, SCR) to 0.145 (SIX, CAR, DCR), as were inbreeding coefficients ranging from 0.148 (RGT, CCR) to 0.174 (DCR).

Principal component analysis showed genetic differentiation with population divergence indicated among some sampled populations although the amount of variation explained was low with only 3.22% on the first axis and 3.08% on the second axis (Figure 3). Population CCR is separated along the first axis and population NCR shows a slight degree of separation along the second axis. Clustering results identified three peaks using the ΔK metric (Evanno *et al.* 2005), the highest peak at K = 2, a second peak at K = 9 and a third smaller peak at K = 4 (Figure S1). The two genetic clusters (Figure 4a) showed similar distributions of each cluster across populations. The four genetic clusters reflected the PCA results, with populations CCR and NCR each having a unique genetic cluster present. The genetic cluster in NCR is also present to a smaller degree in a few individuals of RGT, reflecting the overlap seen in the PCA plot. The other two genetic clusters occur across all populations (Figure 4b). The nine genetic clusters were more aligned with populations, with geographically close populations SIX/SCR and CAR/RRR each sharing large portions of a genetic cluster (Figure 4c). Overall admixture is high amongst populations, again suggesting high levels of gene flow or common ancestry. Populations RGT and CCR show slightly less admixture than other populations, supporting the PCA results.

Overall corrected species genetic differentiation ($F_{STp}$) was found to be 0.0247. Supporting the results of the PCA, populations have very low levels of genetic differentiation. Measures of pairwise population $F_{ST}$ (Table 3, lower triangle) were all found to be significant ($p<0.05$) and ranged from a low of 0.007 between populations DCR and CAR to a high of 0.045 between populations NCR and CCR which were also the populations most differentiated in the PCA. Despite its location geographically midway but most southerly in the species' range, population CCR is most differentiated from all populations with the paired values generally higher than between the other populations. The number of fixed differences between populations ranged from 0 to a high of 3 between populations (SCR and S1T and CAR) (Table 3, upper triangle). Investigation into IBD using a Mantel test found that the relationship between genetic and geographic distance was not significant (Mantel statistic = 0.237, Significance 0.13).

## Discussion

The objective of this study was to acquire baseline information on the level and distribution of genetic variation in *Eucalyptus mackintii* and identify the implications for management of a species subject to disturbances including fire. The SNP dataset provides practical insight into gene flow within the taxon and suggests high levels of genetic connectivity across the

**Table 3. Fixed differences between populations of *Eucalyptus mackintii* (upper triangle), Pairwise genetic distance amongst populations ($F_{ST}$) (lower triangle). Higher values indicate greater differentiation.**

| Population | RGT | NCR | SIX | SCR | CCR | S1T | CAR | DCR | RRR |
|---|---|---|---|---|---|---|---|---|---|
| **RGT** | - | 1 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **NCR** | 0.029 | - | 0 | 0 | 1 | 2 | 1 | 0 | 1 |
| **SIX** | 0.022 | 0.03 | - | 0 | 0 | 1 | 2 | 0 | 0 |
| **SCR** | 0.025 | 0.032 | 0.014 | - | 2 | 3 | 3 | 0 | 0 |
| **CCR** | 0.037 | 0.045 | 0.032 | 0.038 | - | 0 | 1 | 0 | 0 |
| **S1T** | 0.022 | 0.03 | 0.019 | 0.022 | 0.031 | - | - | 0 | 0 |
| **CAR** | 0.026 | 0.028 | 0.023 | 0.016 | 0.034 | 0.014 | - | 0 | 0 |
| **DCR** | 0.027 | 0.023 | 0.02 | 0.026 | 0.028 | 0.012 | 0.007 | - | 0 |
| **RRR** | 0.021 | 0.034 | 0.022 | 0.021 | 0.041 | 0.017 | 0.012 | 0.012 | - |

species range. However, the landscape patterns revealed require some qualification as the sampled trees are likely to be several decades old, so the conditions under which seedlings germinated and that maturing plants were subject to may differ from contemporary conditions. Quantification of genetic diversity in contemporary seedling recruits is needed to confirm whether those genetic patterns are being maintained. The landscape genetic patterns among populations of *E. mackintii* are a useful guide for management. Including genetic information in management strategies has a role in ensuring that the genetic integrity of a rare species can be maintained (Ellstrand & Rieseberg 2016). Given the low percentage variation accounted for by the PCA, we anticipate that other unexplored factors such as environmental conditions and/or species demographic

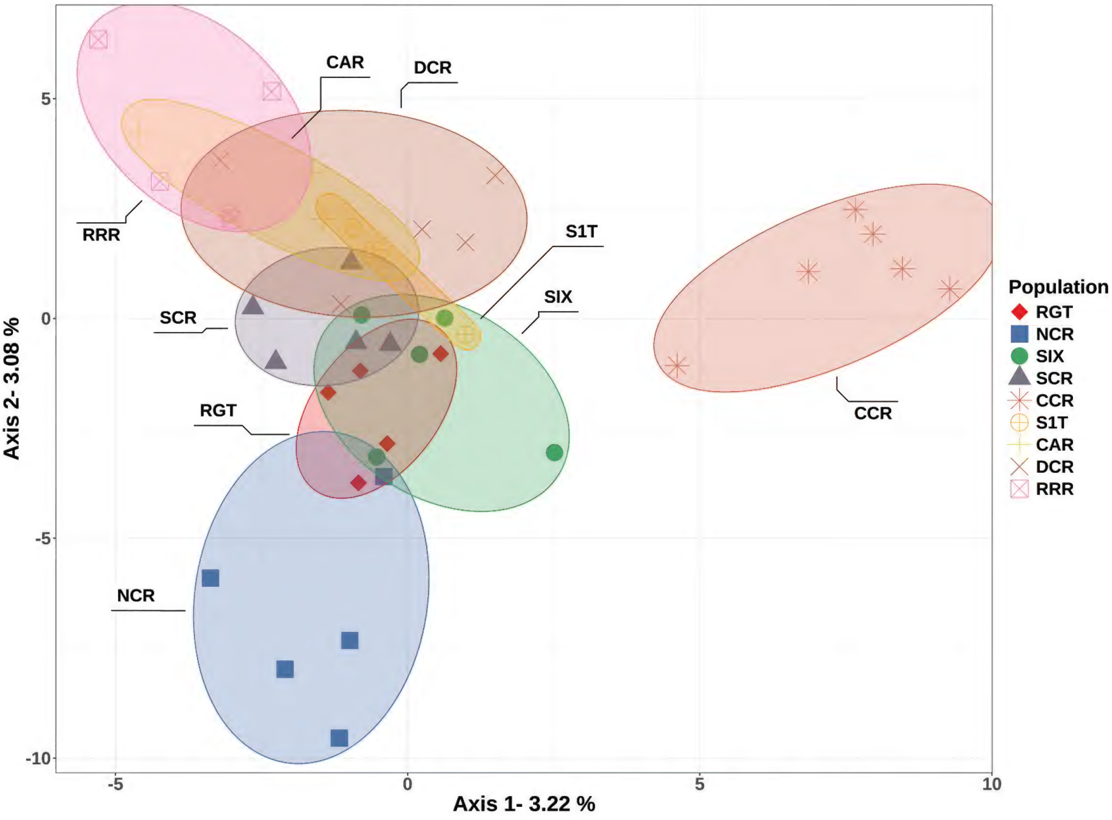

**Figure 3** Principal component analysis of genetic differentiation.

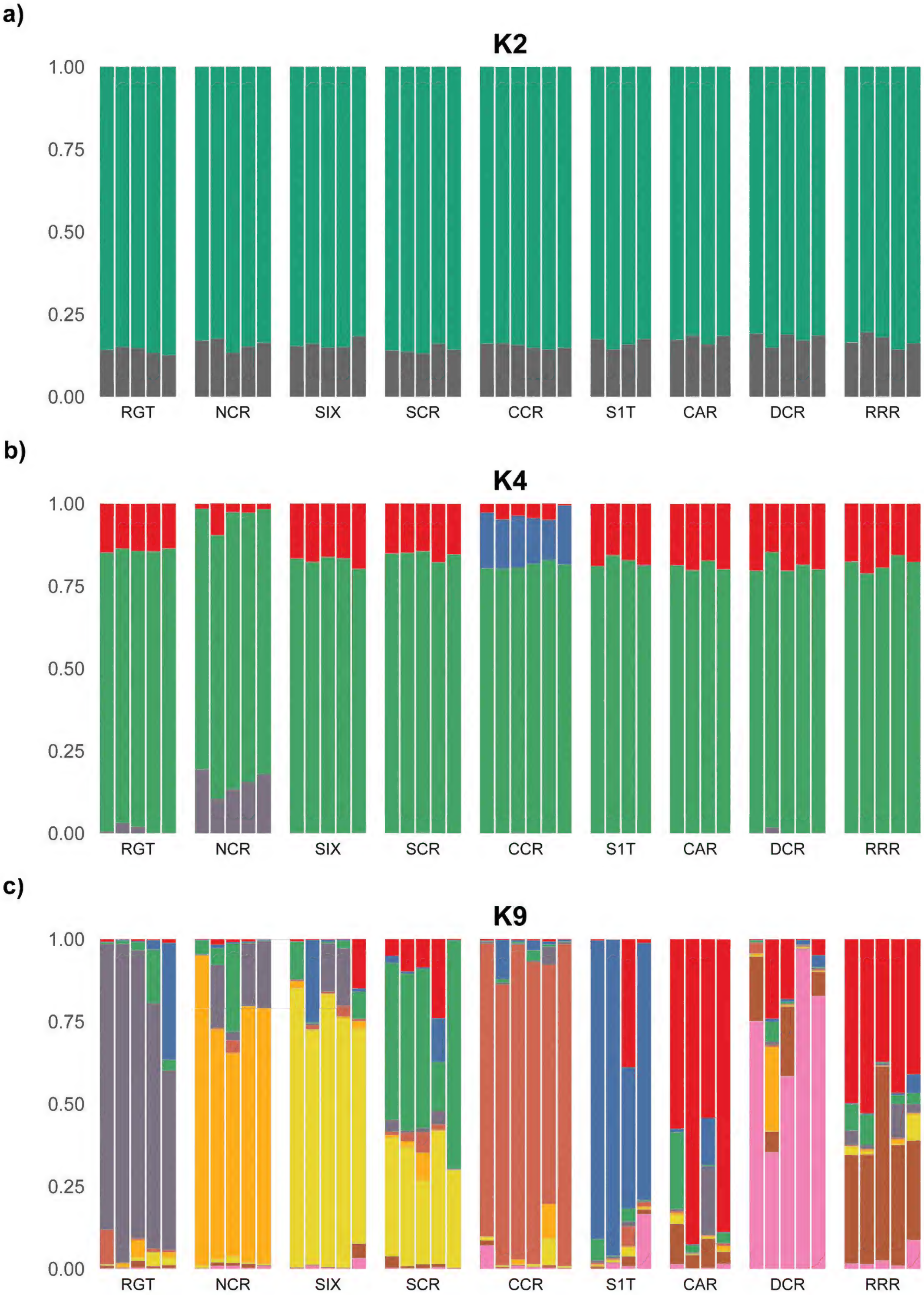

**Figure 4** Genetic clustering results from *Eucalyptus mackintii*. Structure analysis for two clusters (a), four clusters (b) and nine clusters (c). Each individual is represented by a vertical bar which is apportioned into its kinship to each of the identified genetic clusters.

history have also influenced the genetic patterns observed.

Several eucalypt species exhibit low genetic structuring and high levels of gene flow, reflecting strong genetic connectivity across their respective landscapes. *Eucalyptus albens* Benth. and *E. sideroxylon* A.Cunn. ex Woolls were both found to have very low $F_{ST}$ values, 0.018 and 0.017 respectively (Murray *et al.* 2019). Genetic differentiation was low between sites across the range of *E. mackintii* (0.0247) which is indicative of a high level of gene flow enabling genetic connectivity. Higher $F_{ST}$ values have been found in eleven *Eucalyptus*, with values found between 0.03 in *E. pauciflora* to 0.127 in *E. cunninghamii* Sweet (Gauli *et al*. 2014; Supple *et al.* 2018; Rutherford *et al*. 2018). Despite the low levels of differentiation found in *E. mackintii*, there were some signals of genetic structuring amongst populations. Our results highlighted population CCR and to a lesser extent NCR as being somewhat genetically distinct, a characteristic not readily apparent from the spatial distribution of sample locations and which may be due to their local demographic histories. While gene flow appears high and genetic differentiation is low, it is still important to conserve the genetic diversity that is present across the range. The genetic diversity results found in *E. mackintii*, observed heterozygosity of 0.189, are similar to that found in eleven *Eucalyptus* species ranging from 0.133 in *E. stricta* Sieber ex Spreng. and *E. laophila* L.A.S.Johnson & Blaxell to 0.246 in *E. langleyi* L.A.S.Johnson & Blaxell (Rutherford *et al*. 2018). With an estimated generation time of 100–150 years and individual longevity of 150–250 years (DELWP 2021b), sampled trees are likely to be several decades old. Therefore, our results are not measures of contemporary genetic patterns meaning that the impact of any recent habitat modifications or environmental change on genetic connectivity may not be apparent for several generations.

Understanding the spatial distribution of a species is essential for effective management, protection, and conservation. While qualified and trained botanists are able to identify species, on-ground managers may have a harder time when there are multiple species that co-occur and have a very similar morphology. There are over 700 species of *Eucalyptus* in Australia and morphological similarity can make it challenging to accurately identify them to the species level, and particular groups can be especially problematic to non-experts (Slee *et al.* 2020). The misidentification of some samples collected as *E. mackintii* underscores the difficulty of identifying this species when it grows sympatrically with one or more other eucalypts, particularly after fire when a number of characteristics may not be available. Adding to this challenge is the propensity for *Eucalyptus* species to hybridise with their congeners, which can produce plants that are variable along a spectrum between parent plants, potentially adding to identification confusion (Potts & Reid 1985; Griffin *et al.* 1988; Neaylon *et al.* 2001; Field *et al.* 2009). At this stage, hybridisation between *E. mackintii* and sympatric stringybarks is not known but hybridity or introgression could be examined genetically by including all sympatric eucalypt species if the inclusion of seed from misidentified trees is likely to affect the conservation value of seed resources.

The resprouting of mature *Eucalyptus mackintii* following the 2019/2020 wildfires shows that the species has some resilience to fire. Resprouting is expected to result in earlier flowering than seedlings, which should assist in providing a more rapid replenishment of the soil seedbank and facilitate new seed collections. Despite these expectations, ongoing field monitoring is an important conservation requirement to enable effective assessment of the recovery of fire-affected populations of *E. mackintii* and the forest ecosystem more generally over time (Gallagher *et al.* 2021). Short fire intervals have been implicated in a failure in the ability of fire-tolerant trees to continually respond by resprouting (Fairman *et al.* 2019) although other studies have shown resilience to repeated canopy fires in vegetation dominated by eucalypts that resprout from epicormic buds (Collins 2020). To support seed production and ensure viable seed collection, fire management strategies should consider maintaining intervals that allow plants to recover and reach reproductive maturity and produce viable seed.

High levels of genetic diversity generally improve the potential of a species to persist under changing conditions (Nicotra *et al.* 2010) and should be captured as representatively as possible in any germplasm collections planned for conservation management. The Victorian Conservation Seedbank currently holds two seed collections for *E. mackintii*. The older collection made in 2007 is from location DCR, north of Orbost. The collection had an initial inferred viability of 100% (cut test) and germination rate of 80% and the high viability was retained in subsequent germination trials (VCS, unpublished data). In contrast, the 2021 seed collection sourced from Bruthen-Nowa Nowa Rd, the same location as an initial genetic collection, had only 10% inferred viability (cut test) and only 12% germination of filled seed. The initial genetic analysis of samples indicated that the leaf collections from this location were taken from more than one eucalypt species, thus samples from that location were removed from all analyses. The seed collection from the Bruthen-Nowa Nowa Rd location requires assessment to confirm the identity of the species held, or should be discarded and collected anew to remove the possibility of inadvertent sampling of non-target eucalypts.

In the limited number of studies available it was found that *ex situ* seedbank collections do not effectively capture the genetic variation of wild populations (Wei & Jiang 2021). Recommendations for optimal seed collecting suggest that when range-wide population structure exists, the spatial distribution of sampling is crucial although the importance is reduced as gene flow amongst populations increases (Hoban & Schlarbaum 2014). Sampling seed from approximately 200 to 300 individuals in total across a species' geographic range may suffice, however, a robust minimum collection for *ex situ* gene conservation will often be 1000 individuals or more to compensate for expected losses over time (Hoban 2019). To maximise the genetic diversity in *ex situ* collections, it would be prudent to collect from multiple locations across the species' range and across different years. This is particularly important for sites where seed quality is found to be low because this may vary from year to year. Each seed collection should be stored separately to provide for future genotype selection. Seed collections are needed to fill the geographic gaps in current collections. These include new collections in the western part of the species' range, such as the Rain Gauge Track (RGT) or Nicholson Creek Rd (NCR) in Alfred-Nicholson State Forest, locations within Colquhoun State Forest SCR, and on the southern (CCR) and eastern limits of the species' range (RRR). Such collections would enhance the genetic diversity of the VCS *ex situ* collections.

The conservation requirements of *Eucalyptus mackintii* may need to be re-assessed if a current ban on commercial forestry in native forest is permanent, thus removing the primary threatening process for the species. The species remains at risk due to climate change, with fire activity projected to increase in southeastern Australia. An increase in fire frequency and intensity may compromise the species' capacity to resprout successfully and replenish the soil seed bank. The greatest challenge to managing this species is likely accurate identification. Due to the risks associated with its narrow range and identification challenges, it is recommended that *ex situ* seed collections are held that represent the full genetic diversity that is present across the range, and that any collections be made by those specifically trained in their identification to ensure that the propagules are collected from the correct species.

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## Supplementary information

Available online via https://rbg.vic.gov.au/science/journal/

**Figure S1** K-value plots for selection using the Evanno method from Structure analysis.

**Table S1** Monthly distribution of herbarium specimens, showing counts of total specimens and those bearing buds, flowers or fruit.

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ROYAL BOTANIC
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# Optimising *ex situ* collections to efficiently conserve genetic variation in *Grevillea jephcottii* (Proteaceae)

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## Abstract

Range-restricted species are highly susceptible to threats that impact the whole species in one event. *Grevillea jephcottii* J.H.Willis occurs on two disjunct ranges within Burrowa-Pine Mountain National Park and was severely impacted by bushfire in 2020. To assist with conservation and to ensure genetically representative *ex situ* collections, genetic assessment of *G. jephcottii* was made. Genetic diversity was found to be highly structured between mountain ranges. Populations along Pine Mountain showed high levels of gene flow, while populations on Burrowa Range were structured across geographic distance. Results were used to guide sampling for a genetically representative *ex situ* living collection until plants *in situ* are mature enough to produce seed for inclusion in the Victorian Conservation Seedbank.

***Keywords:*** DArTseq, fire frequency, obligate seeder, population genetics, restricted range

## Introduction

Geographic range size is a strong predictor of extinction risk (Gaston & Fuller 2009, Le Breton *et al.* 2019) and includes both extent of occurrence (EEO) and area of occupancy (AOO). When either are limited, species are placed at a high risk of extinction particularly when populations are severely fragmented (IUCN 2012). By their very nature, range-restricted species are more susceptible to catastrophic events that impact all populations. Fire is one such disturbance that can be devastating, however, species such as obligate seeders may require occasional disturbance for regeneration (Knox & Morrison 2005). Short range endemics are often confined to specific habitats (AOO) within their small geographic areas (EEO), further increasing their vulnerability to extinction (Le Breton *et al.* 2019). With ongoing land clearing, these specialised plants may lose the already small niche that they inhabit. Fragmentation of populations from habitat loss may also restrict gene flow and the diversity needed to adapt under changing conditions.

Many plants have evolved to deal with the natural incidence of fire in the landscape, however, human impacts on the characteristics of fire can disrupt natural cycles and plant responses (Gallagher *et al.* 2021). The reliance of obligate seeders on fires for regeneration renders them vulnerable to changes in the occurrence and attributes of wildfire (Muir *et al.* 2014; Pausas & Keeley 2014). Increased frequency and intensity of wildfires in southeastern Australia have been linked to climate change

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(Abram *et al.* 2021; Canadell *et al.* 2021). When fire frequency increases, the seedbank of obligate seeders may become exhausted and prevent re-establishment if fires recur before seed production is sufficient to replenish it (Muir *et al.* 2014; Gallagher *et al.* 2021). Even if the seedbank is not exhausted, but rather severely diminished, recruitment could be very limited, thus reducing population sizes and impacting the standing genetic diversity available to contribute to subsequent generations. In contrast, if fire frequency is reduced due to human intervention, species risk not having sufficient chance to regenerate and populations may senesce, no longer contributing to the seedbank (Campbell *et al.* 2012; Pausas & Keeley 2014).

The Global Strategy for Plant Conservation has a target of "at least 75% of threatened species in *ex situ* collections" (Convention on Biological Diversity 2012). *Ex situ* seed banks are a feasible means of preserving large amounts of diverse genetic materials for the long term to support conservation efforts (Erickson & Halford 2020; Offord *et al.* 2021). Living *ex situ* collections may also be useful as short term genetic insurance for seed production if *in situ* reproduction is disrupted, and the only *ex situ* option for plants that cannot be effectively seed banked (Guerrant *et al.* 2014; Wyse *et al.* 2018; Martyn Yenson *et al.* 2024). While seed of a threatened species will ideally be collected spatio-temporally to assure appropriate representation of the genetic diversity of a species (Guerrant *et al.* 2014; Hoban & Strand 2015), the reality is that there are not often sufficient resources available to collect and store representatives of all subpopulations. When extreme events such as bushfires impact a range-restricted species, they can highlight the species' precarious position and reinforce the need for appropriate *ex situ* collections. However, after fire, there may be a lag between recruitment and reproductive maturity, thus delaying seed collection for several years (Plumanns-Pouton *et al.* 2024). During this period the plant is also at greater risk of exposure to a subsequent catastrophic event that raises the likelihood of extinction (Plumanns-Pouton *et al.* 2023). In such circumstances, a living *ex situ* collection propagated vegetatively from standing biomass can act as an insurance population preserving some of the genetic diversity of sub-populations and the species until seed can be banked for future conservation.

*Ex situ* conservation collections aim to preserve the genetic diversity of wild populations with the goal of supporting future *in situ* conservation efforts (Volis 2017). Maintaining genetic diversity is important for the long-term resilience of populations (Sgrò *et al.* 2011). To efficiently and effectively manage for genetic diversity both *in situ* and *ex situ* it is important to first understand the extant levels of genetic diversity and how the genetic diversity is structured across the range (Booy *et al.* 2000). This information can guide sampling for *ex situ* collections to ensure that collections are genetically representative and hold the evolutionary potential for conservation needs (Falk & Holsinger 1991, Hoban & Schlarbaum 2014). Importantly, for species with disjunct distributions, subpopulations may be genetically distinct due to restricted gene flow caused by mate or pollinator limitation (Hoebee *et al.* 2008, Holmes *et al.* 2008) and differences in breeding systems (Caddy & Gross 2006). In addition, dispersal barriers such as limited pollinator range, or environmental or topological features, may prevent seed dispersal (Holmes *et al.* 2009; Nistelberger *et al.* 2015) or establishment of new populations, although unique variation in subpopulations can develop over small geographic distances without obvious disjunctions (Wawrzyczek *et al.* 2023).

The Pine Mountain or Green Grevillea, *Grevillea jephcottii* J.H.Willis, is a rare species endemic to a small area of northeastern Victoria found within Burrowa-Pine Mountain National Park (Department of Climate Change, Energy, the Environment and Water 2023). Increased frequency and intensity of wildfires are considered the major threats to the persistence of *G. jephcottii* (Department of Climate Change, Energy, the Environment and Water 2023). This plant is an example of a species whose limited geographic range makes it vulnerable to catastrophic events such as repeated short-interval or extremely hot wildfires that could encompass the species' entire distribution. This occurred in 2020 when widespread bushfires burnt almost 95% of Burrowa-Pine Mountain National Park and most populations of *G. jephcottii*. As the species is an obligate seeder, with no re-shooting or re-sprouting recorded post-fire, its regeneration is dependent on the presence of a viable soil seedbank and environmental conditions conducive to recruitment after fire. To promote resilience under a changing climate this research assessed genetic diversity and estimated connectivity among the populations of *G. jephcottii* to guide conservation efforts to preserve extant genetic diversity. While our focus was on assessing genetic diversity, an *ex situ* living collection was initiated by applying genetic results to the sampling of three populations.

## Methods

### Study Species

The Pine Mountain Grevillea is a low, dense to tall, spindly shrub 1–3 m high (Figure 1). The species is restricted to a small area in north-east Victoria in the Burrowa-Pine Mountain National Park where it occurs in rocky locations and on steep hillsides in dry sclerophyll forest, on granite, at elevations of about 550–650 m (Olde & Marriott 1995, Makinson 1996, Australasian Virtual Herbarium 2021). Steep, rugged topography segregates populations on Pine Mountain and Burrowa Range, and land clearing further isolates the two ranges. The restricted distribution of Pine Mountain Grevillea is a consequence of its narrow ecological niche on steep, rocky hillsides (see topographic features, Figure 2). The species is listed as endangered under both the *Flora and Fauna Guarantee Act 1988* (Vic) (Makinson 1996; Department of Environment, Land, Water and Planning 2021) and the *Environmental Protection and Biodiversity Conservation Act 1999* (Cth) (EPBC Act 1999) in part due to data deficiency. The species is locally common within this narrow range solely within the Burrowa-Pine Mountain National Park conservation estate but its distribution is discontinuous due to habitat specificity and topography. *Grevillea jephcottii* is taxonomically distinct and accepted, with dense, entire, oblong to narrowly elliptic leaves 10–35 mm long, 1.5–6(–8) mm wide. The lower leaf surface is glabrous (sparsely hairy along midvein on juvenile leaves) and the upper surface often hairy with a shortly recurved leaf margin. The flowers, which appear in late winter to summer as a terminal conflorescence, are pale lemon or green, with a

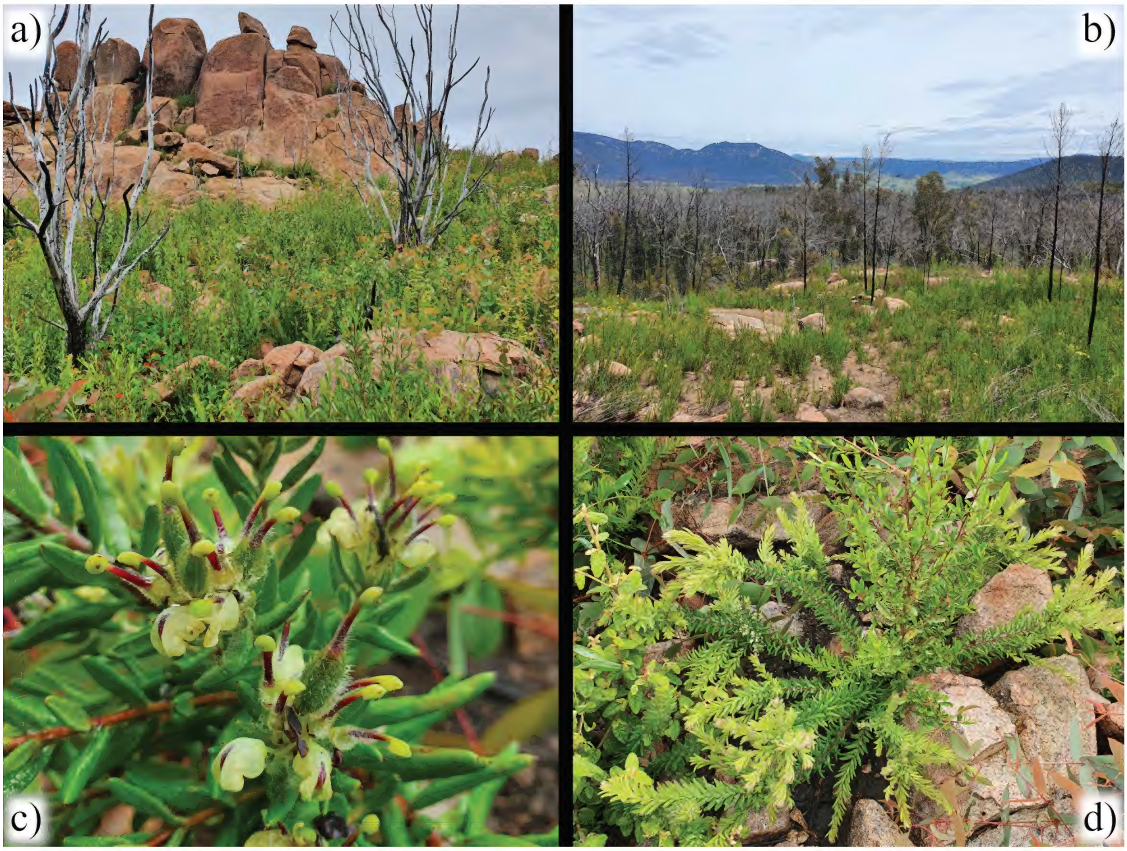

**Figure 1.** (a) Habitat, (b) re-growth after fire, (c) flowers and fruit, and (d) seedling of *Grevillea jephcottii*.

purple style, white hairs and a contrasting green pollen presenter, with pollination most likely effected via birds (Makinson 1996). Subsequent fruits are pilose follicles (Figure 1). The breeding systems of *Grevillea* species are highly variable (Hermanutz *et al.* 1998) although the realised mating systems for many species are predominantly outcrossing. The reproductive biology of *G. jephcottii* has not been investigated to date but the species is considered to be an obligate seeder. The species has been in cultivation for many years, propagating readily from cuttings, and can be grown in a variety of conditions and climates (Hitchcock 2005).

## Sample collection

Population sampling of Pine Mountain Grevillea was performed in November 2021 at locations based on National Herbarium of Victoria records, the Victorian Biodiversity Atlas, and other citizen science records retrieved from the Atlas of Living Australia (Atlas of Living Australia 2021). The spatial relationship among populations is represented in Figure 2 and locations are given in Table 1. Where the widespread 2020 Black Summer Fires affected the species, seedlings of *G. jephcottii* were observed in abundance post-fire but no resprouting or re-shooting was observed. Leaf samples were collected from 5 individuals at each of 13 locations selected across the range of the species. Three sampling locations were unburnt: amongst large boulder refugia 1.6 km and 3.1 km along Pine Mountain Track (PM_TR2 & PM_TR4) and a sheltered location past an old mine site (PM_OMS). At these three locations, samples were collected from adult plants, while samples at all other locations were seedlings. Along the Pine Mountain walking track plants are common but not continuously distributed so sampling locations were selected based on geographic complexity, such as the occurrence on opposite sides of major outcrops or minor summits (Figure 2).

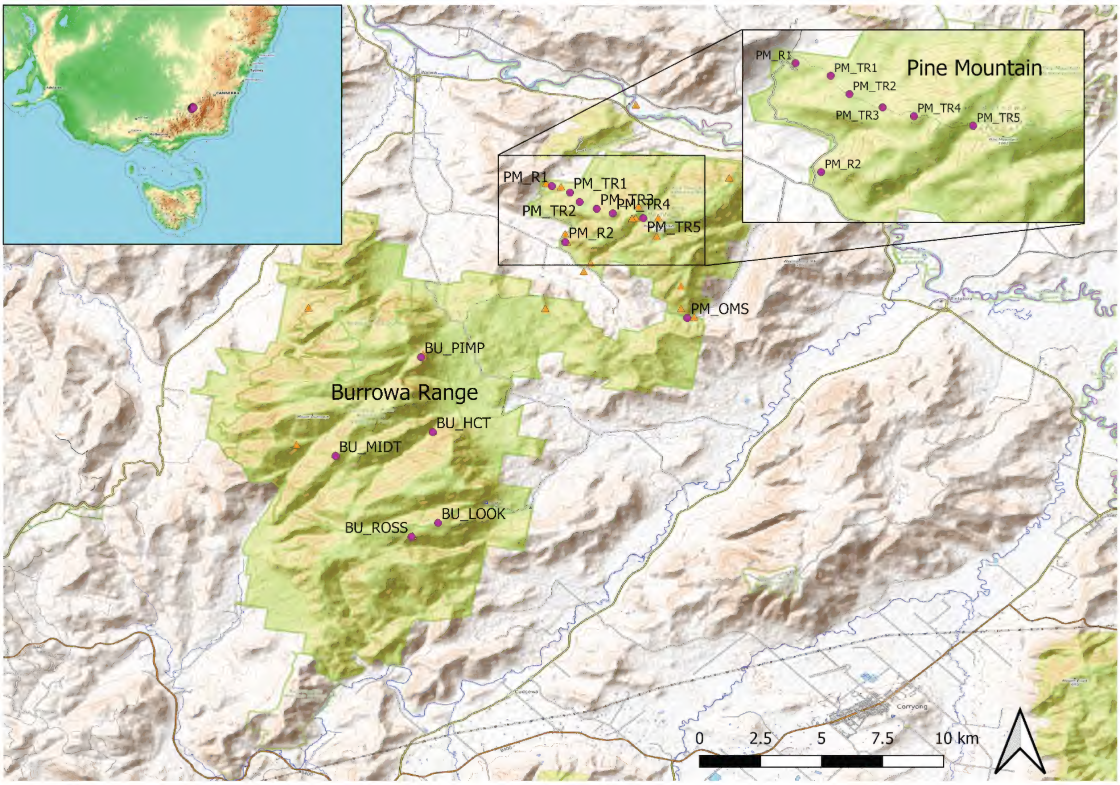

**Figure 2.** Locations of sampled populations (purple circles) and herbarium records (yellow triangle) of *Grevillea jephcottii*. Inset shows the location of study site in Australia: a) shows the site locations and the vegetation remaining across the landscape, b) shows the topography of the area indicating the steep terrain. Map created using QGIS (QGIS Development Team 2025), herbarium records from Atlas of Living Australia (cultivated and spatial uncertainty greater than 2 km removed).

**Table 1. Location details for *Grevillea jephcottii* sample sites.**

| Site | Latitude | Longitude | Location |
|---|---|---|---|
| BU_MIDT | -36.104 | 147.706 | Burrowa Mid tops |
| BU_ROSS | -36.134 | 147.734 | Burrowa Ross lookout |
| BU_PIMP | -36.068 | 147.738 | Burrowa The Pimple |
| BU_HCT | -36.095 | 147.742 | Burrowa Hinces Creek Track |
| BU_LOOK | -36.128 | 147.744 | Burrowa Lookout track |
| PM_R1 | -36.005 | 147.786 | Pine Mountain Pine Mountain Track Road near walking track |
| PM_R2 | -36.025 | 147.791 | Pine Mountain Pine Mountain Track Road near intersection with Sandy Creek Rd |
| PM_TR1 | -36.007 | 147.792 | Pine Mountain Pine Mountain Track 1 km along walking track |
| PM_TR2 | -36.010 | 147.796 | Pine Mountain Pine Mountain Track 1.6 km along walking track |
| PM_TR3 | -36.013 | 147.802 | Pine Mountain Pine Mountain Track 2.5 km along walking track |
| PM_TR4 | -36.015 | 147.808 | Pine Mountain Pine Mountain Track 3.1 km along walking track |
| PM_TR5 | -36.016 | 147.819 | Pine Mountain Pine Mountain Track 4.5 km along walking track |
| PM_OMS | -36.053 | 147.836 | Pine Mountain Past the old Mine Site |

## DNA extraction, amplification and sequencing

Approximately 10 mg from each of the 65 silica-dried leaf samples were sent to the commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq™ analysis, a reduced representation sequencing method (Kilian *et al.* 2012). DNA was extracted at DArT™ using the Nucleo Mag Plant C-Bead kit (Machery Nagel, Germany), on a Tecan 100 platform following the manufacturers protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and MseI and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina NovaSeq X+ sequencer. Filtering of poor-quality sequences (Phred score <30) and read assembly were undertaken by DArT's proprietary primary analytical pipeline and the filtered, assembled data were then used in a proprietary secondary pipeline for SNP calling (DarTsoft14).

## Data analysis

Results received from DArTseq were filtered using the R packages dartR (Gruber *et al.* 2018; Mijangos *et al.* 2022) and Poppr (Kamvar *et al.* 2014, 2015) in R (R Core Team 2024). The data were filtered to a locus call rate of 0.95, an individual call rate of 0.9, a reproducibility threshold of 0.95, a minor allele frequency greater than 4, removal of monomorphic loci, removal of secondaries, a read depth between 5 and 100, and filtered on Hamming distance to remove potential paralogues.

To identify genetic clusters of individuals and visualise the major axes of variation between clusters, principal component analysis (PCA) was undertaken, implemented in the adegenet package (Jombart 2008; Jombart & Ahmed 2011) in R (R Core Team 2024). Expected and observed heterozygosity, inbreeding coefficients and pairwise population differentiation ($F_{ST}$) were assessed using the adegenet (Jombart 2008; Jombart & Ahmed 2011), hierfstat (Goudet & Jombart 2020), dartR (Gruber *et al.* 2018; Mijangos *et al.* 2022) and Poppr (Kamvar *et al.* 2014, 2015) packages in R. Analysis of molecular variance (AMOVA) was tested using the Poppr (Kamvar *et al.* 2014, 2015) package in R. Isolation by distance and fixed difference analysis were assessed using R package dartR (Gruber *et al.* 2018; Mijangos *et al.* 2022). Population genetic structure was also explored using Structure 2.3.4 (Pritchard *et al.* 2000) using K-values from 1 to 15. Ten independent runs were undertaken for each K value with a burnin of 400,000 and 600,000 MCMC iterations. The R package pophelper (Francis 2017) and ggplot2 (Wickham 2016) were used to visualise results and select the optimal K based on the ΔK metric (Evanno *et al.* 2005).

## Results

The results from DArTseq contained 64 samples and 170,648 loci; one individual failed sequencing. After filtering, the filtered SNP data set contained 15,856 loci and 63 individuals across 13 populations; one individual was removed during filtering. *Grevillea jephcottii* was found to have an average observed heterozygosity across all populations of 0.103 and expected heterozygosity of 0.131. Individual population genetic diversity values are shown in Table 2. The observed heterozygosity values ranged from 0.119 to 0.135, with population BU_PIMP having the lowest value and population BU_ROSS having the highest value. Expected heterozygosity levels were consistent across populations. Observed heterozygosity was lower in every population than expected heterozygosity. This is reflected in the inbreeding coefficient, with several populations (PM_R2, BU_PIMP, BU_MIDT) showing high levels of inbreeding. The number of fixed differences between populations ranged from 0 to 16 (Table 3). The highest number of fixed differences (16) was found between populations PM_TR3 and PM_TR4 that are ~600 m apart.

**Table 2. Genetic diversity characteristics of the *Grevillea jephcottii* populations (standard error in brackets), * indicates sampled population was unburnt.**

| Population | # Individuals | Expected heterozygosity | Observed heterozygosity | Inbreeding coefficient |
|---|---|---|---|---|
| PM_R1* | 5 | 0.123 (0.001) | 0.114 (0.002) | 0.058 |
| PM_R2* | 4 | 0.128 (0.001) | 0.095 (0.001) | 0.213 |
| PM_TR1* | 5 | 0.132 (0.001) | 0.114 (0.002) | 0.108 |
| PM_TR2 | 5 | 0.129 (0.001) | 0.113 (0.002) | 0.099 |
| PM_TR3* | 5 | 0.132 (0.001) | 0.112 (0.001) | 0.122 |
| PM_TR4 | 5 | 0.125 (0.001) | 0.107 (0.001) | 0.115 |
| PM_TR5* | 5 | 0.129 (0.001) | 0.1 (0.001) | 0.178 |
| PM_OMS | 5 | 0.129 (0.001) | 0.097 (0.001) | 0.194 |
| BU_PIMP* | 4 | 0.119 (0.001) | 0.079 (0.001) | 0.278 |
| BU_HCT* | 5 | 0.124 (0.001) | 0.104 (0.001) | 0.126 |
| BU_MIDT* | 5 | 0.121 (0.001) | 0.078 (0.001) | 0.296 |
| BU_LOOK* | 5 | 0.127 (0.001) | 0.109 (0.001) | 0.117 |
| BU_ROSS* | 5 | 0.135 (0.001) | 0.115 (0.001) | 0.111 |

Principal component analysis showed the differentiation among sampled populations (Figure 3). The strongest split is between populations on Pine Mountain and populations on Burrowa Range, accounting for the differentiation seen in axis 1 (4.8%). The second axis identifies the variation amongst the Burrowa Range populations which cluster spatially with BU_HCT being distinct. The third axis explains a similar amount of variation as the second axis, showing the East to West spread of variation amongst the Pine Mountain populations.

Overall species genetic differentiation ($F_{ST}$) was found to be 0.1075. Measures of between population $F_{ST}$ ranged from a low of 0.056 to a high of 0.177 (Table 3). The highest differentiation was found between

**Table 3. Pairwise genetic distance amongst populations of *Grevillea jephcottii* (upper triangle), higher values indicate greater differentiation. Geographic distances (km) between populations (lower triangle).**

| Population | PM_ R1 | PM_ R2 | PM_ TR1 | PM_ TR2 | PM_ TR3 | PM_ TR4 | PM_ TR5 | PM_ OMS | BU_ PIMP | BU_ HCT | BU_ MIDT | BU_ LOOK | BU_ ROSS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PM_R1 | - | 0.109 | 0.077 | 0.099 | 0.097 | 0.138 | 0.118 | 0.123 | 0.151 | 0.177 | 0.154 | 0.165 | 0.138 |
| PM_R2 | 2.3 | - | 0.069 | 0.093 | 0.07 | 0.106 | 0.084 | 0.093 | 0.109 | 0.147 | 0.117 | 0.131 | 0.113 |
| PM_TR1 | 0.7 | 2 | - | 0.056 | 0.058 | 0.098 | 0.079 | 0.088 | 0.116 | 0.139 | 0.115 | 0.124 | 0.103 |
| PM_TR2 | 1.1 | 1.7 | 0.5 | - | 0.075 | 0.114 | 0.099 | 0.101 | 0.132 | 0.155 | 0.133 | 0.143 | 0.121 |
| PM_TR3 | 1.8 | 1.7 | 1.1 | 0.6 | - | 0.084 | 0.071 | 0.079 | 0.111 | 0.136 | 0.109 | 0.119 | 0.098 |
| PM_TR4 | 2.3 | 2 | 1.7 | 1.2 | 0.6 | - | 0.097 | 0.11 | 0.139 | 0.163 | 0.136 | 0.147 | 0.126 |
| PM_TR5 | 3.3 | 2.8 | 2.6 | 2.2 | 1.6 | 1 | - | 0.085 | 0.116 | 0.143 | 0.115 | 0.129 | 0.106 |
| PM_OMS | 7 | 5.1 | 6.4 | 5.9 | 5.4 | 4.9 | 4.3 | - | 0.113 | 0.141 | 0.113 | 0.127 | 0.103 |
| BU_PIMP | 8.2 | 6.7 | 8.3 | 8.2 | 8.4 | 8.7 | 9.3 | 9 | - | 0.153 | 0.105 | 0.132 | 0.112 |
| BU_HCT | 10.8 | 8.9 | 10.8 | 10.6 | 10.6 | 10.7 | 11.2 | 9.6 | 3.1 | - | 0.141 | 0.147 | 0.13 |
| BU_MIDT | 13.2 | 11.6 | 13.3 | 13.2 | 13.3 | 13.5 | 14.1 | 13 | 4.9 | 3.4 | - | 0.125 | 0.105 |
| BU_LOOK | 14.3 | 12.2 | 14.2 | 13.9 | 13.9 | 13.9 | 14.2 | 11.8 | 6.8 | 3.7 | 4.4 | - | 0.077 |
| BU_ROSS | 15.1 | 13.1 | 15 | 14.8 | 14.7 | 14.8 | 15.1 | 12.8 | 7.3 | 4.3 | 4.2 | 1 | - |

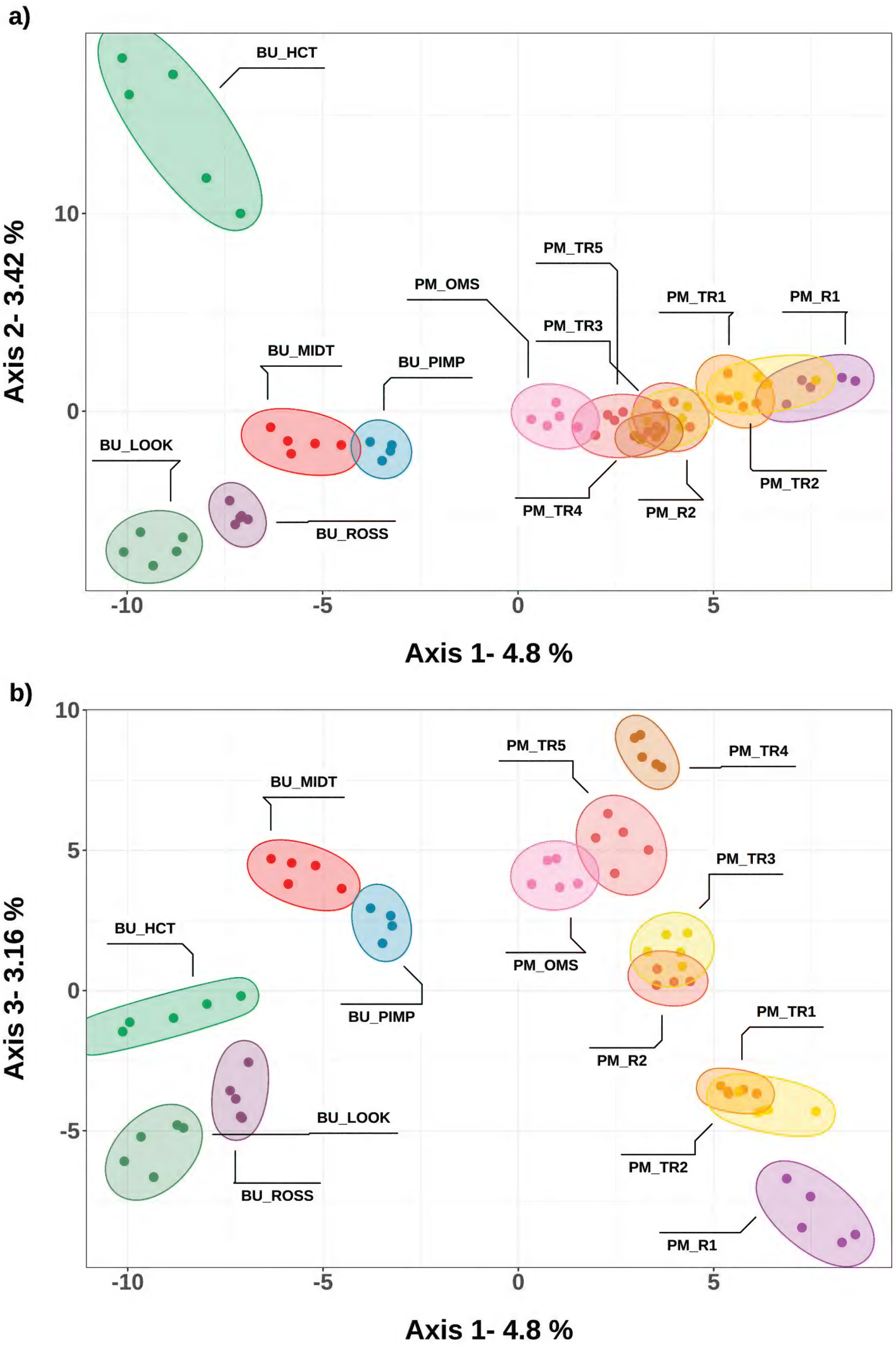

**Figure 3.** Principal component analysis of genetic differentiation for 13 populations of *Grevillea jephcottii* based on the SNP dataset of 15,856 loci and 63 individuals.

populations PM_R1 and BU_HCT which are located on separate mountain ranges, and the lowest differentiation was found between populations PM_TR1 and PM_TR2, the geographically closest populations. The lowest value between populations in the Burrowa Range was 0.077 between populations BU_LOOK and BU_ROSS, the closest populations on the Burrowa Range. These results support the findings of the genetic diversity and principal component analysis with population BU_HCT in the Burrowa Range showing the greatest differentiation from all other populations. Levels of differentiation amongst populations on Pine Mountain ranged from 0.056 to 0.138, average of 0.092. Levels of differentiation amongst populations on Burrowa Range were generally higher than those amongst populations on Pine Mountain (0.077–0.153), average of 0.123. The isolation by distance result confirms these findings, showing a significant association between geographic and genetic distance (Mantel statistic = 0.557, Significance = 0.0003). Analysis of molecular variance (AMOVA) showed that the majority of genetic variation was found within samples with 70% of the variation explained, while only 11.5% of the variation was explained by the genetic difference between populations and the remainder apportioned between samples within populations (*p* =0.001).

Population genetic structure, assessed in Structure, identified 12 genetic clusters across the 13 populations sampled (Figure 4) (Figure S1). These results show the finer detail of groupings identified in the PCA. Most populations are represented predominately by a single genetic cluster, the exception being populations PM_TR1 and PM_TR2 which share a genetic cluster and show admixture with populations PM_R1 and PM_TR3. The levels of admixture between the Pine Mountain genetic clusters indicate that there is gene flow between close populations, as indicated in the isolation by distance results whereas populations on Burrowa Range generally show less admixture with a small amount indicated only between populations BU_LOOK and BU_ROSS.

## Discussion

The genetic assessment of *G. jephcottii* identified that genetic diversity is highly structured between the two mountain ranges, with each range showing different patterns of genetic structure. This has implications for

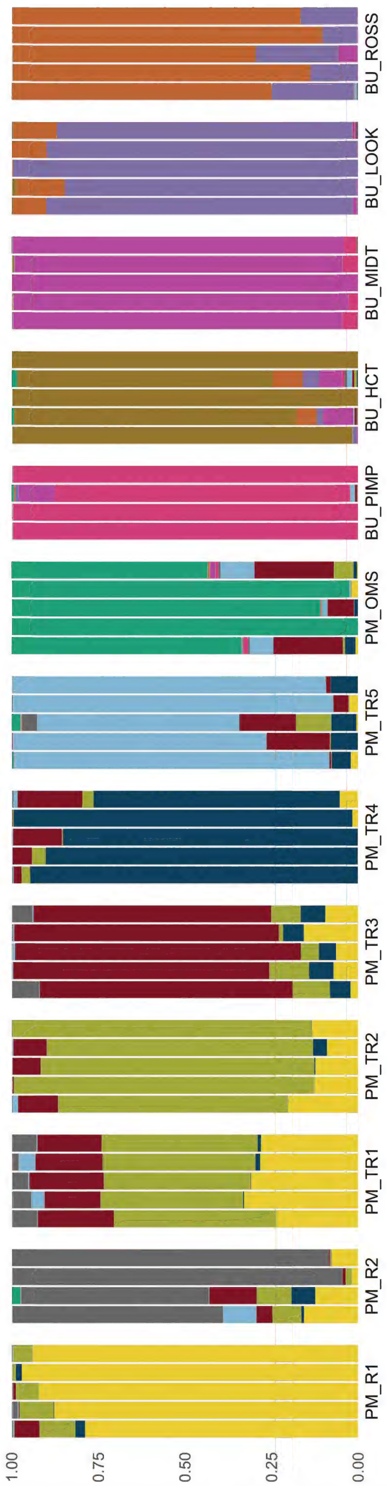

**Figure 4.** Genetic clustering results from Structure analysis (K = 12) for 13 populations of *Grevillea jephcottii*. Each individual is represented by a horizontal bar that is apportioned into its kinship to each of the 12 identified genetic clusters.

the collection and storage of germplasm to ensure that *ex situ* holdings encapsulate the available diversity (Guerrant *et al.* 2014, Hoban & Schlarbaum 2014, Offord *et al.* 2021). The scattered distribution along Pine Mountain showed evidence of gene flow amongst neighbouring subpopulations, while populations on Burrowa Range were found to be more structured with geographic distance a good estimate of genetic differentiation. These results provide a valuable contribution to the conservation of *G. jephcottii*. By targeting germplasm collection with knowledge of the underlying genetic diversity and landscape distribution, it becomes possible to use specific collections to augment genetic diversity *in situ* or to experimentally manipulate seed genetics and production *ex situ*.

While regeneration from seed was observed at fire affected sites, the time required for these small plants to reach sexual maturity is unknown. A risk for the species is that the pre-fire seedbank may have been completely depleted at burnt sites or frequent repeat burning in future could exhaust seedbanks and prevent population re-establishment if fires recur before seed production is sufficiently high (Auld & Denham 2006). A reasonable time for replenishment of seedbanks for woody species is 15 years (Auld *et al.* 2022). The large numbers of *G. jephcottii* seedlings seen at all burnt areas indicate a healthy soil seed bank prior to the recent fire but no indication of what remains. Considering all adult plants are killed by fire it is imperative but not guaranteed that these new seedlings have conditions favourable for persistence (Auld *et al.* 2022). Fresh seed from species that require disturbance for germination can have a dormancy that needs to be broken before germination (Pickup *et al.* 2003). Environmental conditions vary from year to year and factors such as temperature influence traits like phenology, bud set and growth, and these changes can subsequently affect the seed production of individual plants (Solvin & Steffenrem 2019). Therefore, it is important to allow for spatio-temporally variable seed to be incorporated into the seedbank to ensure that the genetic diversity of the soil seedbank and any populations derived from it have the best chance of survival and persistence. By combining seed from multiple years, soil seedbanks can accumulate and preserve the genetic diversity of standing populations (Honnay *et al*. 2008, Ottewell *et al*. 2011, Templeton & Levin 1979). This was seen in the results of this study with heterozygosity values similar across burnt and unburnt sites, suggesting a genetically diverse seed bank prior to the fires.

Inbreeding levels in all populations of *G. jephcottii* indicate restricted gene flow and dispersal limitations for the species, findings which are confirmed by the correlation between genetic and geographic distance in the isolation by distance analysis. On Pine Mountain, the species is locally common with populations discrete and scattered across the mountain; as such we did not expect to see restricted gene flow amongst these geographically close populations. Populations that are not geographically isolated are usually expected to maintain genetic connectivity; thus, the results are likely an indication of restricted gene flow that may be a consequence of short distance seed dispersal (Ellstrand & Elam 1993, Ellstrand 2014), limited pollinator movement between subpopulations, and/or possible self-compatibility and selfing.

The genetic results of this study provide a valuable contribution to the conservation of *G. jephcottii* and have been of practical use to design seed collection strategies and guide *in situ* sampling of plants to establish a living insurance collection until it is possible to collect seed. Prior to the 2020 wildfires, the Victorian Conservation Seedbank (VCS) did not hold any seed collections for the Pine Mountain Grevillea. It is important that seed production is sufficient to allow replenishment of the *in situ* seedbank before collecting seed for conservation (Gallagher *et al.* 2022) and it may be some years before seed is produced in *G. jephcottii* populations and in sufficient quantities. Therefore, following initial results from this genetic analysis, cultivated *ex situ* collections were assembled from plants at Pine Mountain Track (PM_PMT), The Pimple (BU_PIMP) and Hinces Creek Track (BU_HCT) with the potential to be used for seed production. In 2024, a small collection of seed was made along Pine Mountain Track for the VCS, the beginning of a seed bank collection for the species, and additional collections will be made as seed production increases. To fully capture the genetic diversity present in *G. jephcottii* we would recommend that seed collections be made from each of the populations on the Burrowa Range and from a number of locations spread across Pine Mountain. We acknowledge that collecting samples from every population may not be feasible, especially when establishing living collections that require substantial space and ongoing maintenance, each with associated costs. Under such constraints, we propose that both the Pine Mountain and Burrowa Range be represented to capture the two main genetic groups, with the aim being to make a few collections from the distribution on Pine Mountain (e.g. PM_OMS, PM_TR3 and PM_R1) and a few from Burrowa Range (i.e. BU_HCT, BU_ROSS or BU_LOOK and BU_PIMP or BU_MIDT). Due to the higher inbreeding at PM_R2, BU_PIMP, and BU_MIDT, *in situ* efforts could promote reconnecting these populations with those close by, likely an easier option at PM_R2. An alternate option would be to augment isolated populations such as BU_PIMP and BU_MIDT with a small number of plants from neighbour populations to increase the diversity at those sites. By introducing only a small number of plants, the risk of any negative outbreeding effects would be minimised while still effecting an increase in genetic diversity.

While these recommendations align with standard conservation practices, particularly by collecting material throughout the distribution and from geographically disjunct populations (Offord *et al.* 2021), the genetic results confirm that populations on Burrowa Range are both geographically and genetically isolated. In such remote and rugged landscapes where the true extent and location of populations may be uncertain, genetic data can serve as a valuable proxy. It can also help infer the presence of undocumented populations that may act as genetic stepping stones to maintain connectivity. The genetic dataset generated by this study can be used as a baseline comparison for future genetic monitoring to estimate the population genetic impacts of fires on *G. jephcottii* and for the management of genetic resources both *in situ* and *ex situ*.

## Acknowledgements

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## Supplementary information

Available online via: https://www.rbg.vic.gov.au/science/journal/

**Figure S1** K-value plots for selection using the Evanno method from Structure analysis.

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# Conservation of two subspecies of *Olearia rugosa* (Asteraceae) post-bushfire complicated by occasional hybridisation

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## Abstract

This study provides an overview of genetic diversity in populations of *Olearia rugosa* (F.Muell. ex Archer) Hutch. subsp. *angustifolia* Hawke ex Messina and subsp. *distalilobata* Hawke ex Messina, two of four Victorian subspecies, to assist in the development of a conservation framework. Populations of both subspecies burnt in the Black Summer bushfires (2019/2020) are genetically distinct from the two other Victorian subspecies. Hybridisation was confirmed at one site where *O. rugosa* subsp. *angustifolia* grows sympatrically with *O. lirata* (Sims) Hutch. and a morphological intergrade occurs there. Field identification based on morphology was inconsistent with genetic identity of species, subspecies and hybrids. *In situ* and *ex situ* genetic management including seed collection and genetic rescue require prior assessment of plant genetic status, particularly if there is a risk of hybridisation between taxa.

***Keywords:*** isolated populations, hybridisation, *Olearia lirata*, DArTseq

## Introduction

Geographical isolation and subsequent lack of gene flow between populations represents a major speciation factor in plants (Rieseberg & Willis 2007, Ellstrand 2014). Subspecies rank is commonly used in plant taxonomy to represent geographically isolated populations that exhibit significant and recognisable morphological differences, while remaining insufficiently distinctive to merit species status and retaining the potential to interbreed (Hamilton & Reichard 1992, Stajsic 2010, Messina *et al*. 2013, 2014). Despite some dispute about the legitimacy of accepting the rank of subspecies (Burbrink *et al.* 2022), their recognition can assist in promoting policy around conserving evolutionarily significant units (Braby *et al.* 2012) although the two are not necessarily equivalent. Genetic differentiation can be detectable between subspecies (Robins *et al*. 2021, Blyth *et al.* 2021, Sampson & Byrne 2022) and may be correlated with some reproductive isolation that may bring about speciation (Stankowski & Ravinet 2021). Genetic assessment is valuable for shaping conservation actions for species to ensure the unique genetic diversity of each taxon is recognised and preserved (Monks *et al.* 2022). This is particularly important if there are life strategies that differ between subspecies as this may impact how habitats are managed (Millar *et al.* 2010). Despite the value of genetic information in managing conservation targets, genetic evaluations have been included in only 52% of Australian species recovery plans (Pierson *et al*. 2016).

Hybridisation plays a significant role in speciation in many plant families including Asteraceae (Wong *et al*. 2022), both over evolutionary

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timeframes (Rieseberg *et al.* 2003) and in recent decades (Lowe and Abbott 2004, Abbott *et al.* 2009). Intra- and inter-specific hybridisation is common in plants (Griffin *et al*. 1988, Whitney *et al*. 2010) and has been observed to increase with environmental disturbance, whether natural (Stebbins 1950) or human-mediated, including the introduction of non-native species (Potts *et al.* 2003, Laikre *et al.* 2010, Larcombe *et al.* 2016). Negative risks of hybridisation include the loss of currently defined parental species identity and genetic uniqueness (Allendorf *et al.* 2001). For example, the introduced invasive species *Senecio madagascarensis* hybridises with the Australian native species *S. pinnatifolius,* the identity of which may come under threat if the presence of *S. madagascariensis* increases in areas of contact (Prentis *et al.* 2007, Dormontt *et al.* 2017). There is also the risk of outbreeding depression and genetic swamping with hybridisation, due to the disruption of local adaptation and decreased fitness in early generation hybrids (Lopez *et al.* 2000, summarised in Todesco *et al.* 2016). However, this must be balanced with the risks of inbreeding depression and a lack of genetic diversity that may impede adaptive potential and resilience, both of which could elevate the extinction risk for species in highly modified, altered, degraded and fragmented environments (Frankham 2015, Chan *et al.* 2019). Genetic traits gained through hybridisation can benefit a species by expanding its environmental tolerances and enabling greater ecological divergence than that of the parent species (e.g. *Helianthus*, Rieseberg *et al.* 2003) and may be beneficial for persistence under climate change (Vallejo-Marín *et al.* 2016, Pfeilsticker *et al.* 2022).

The evolutionary influence of hybridisation is variable (Dormontt *et al.* 2017, Mitchell *et al.* 2022) and the drivers of hybrid zones are still unknown for most species (Abbott 2017). Some authors have argued that novel hybrids may have a high conservation value because of their significance in evolution (Thompson *et al.* 2010), however, the conservation of novel hybrids and hybrid populations is complex (Muniz *et al.* 2020). In Australia, Commonwealth and state legislation do not afford consistent protection for hybrids (e.g. transient hybrids are afforded protection under the Victorian Government *Flora and Fauna Guarantee Act 1988* (FFG Act 1988) but not the Commonwealth *Environment Protection and Biodiversity Conservation Act 1999* (EPBC Act 1999)). Similarly, conservation of hybrids may have detrimental flow-on effects for the conservation of native species where hybridisation with a foreign species result in new, fertile, stable hybrid taxa (Lowe & Abbott 2004, Abbott *et al.* 2009) rather than a transient hybrid entity (van Dijk *et al.* 2020). Moreover, hybridisation in plants can also impinge on the effectiveness of the conservation management of 'pure species' if not detected (Fatemi & Gross 2009), particularly in *ex situ* collections aimed to conserve the genetic distinctiveness and diversity of a species concept.

The genus *Olearia* as traditionally recognised, comprises ca. 180 species and is the most speciose genus of Asteraceae in Australia where all 130 species are endemic (VicFlora 2025). Unlike the majority of New Zealand species, the Australian taxa are noted for being largely diploid (2n=18), though there are exceptions, such as the *Olearia phlogopappa* (Labill.) DC. complex which is tetraploid (2n=36, Watanabe *et al.* 1996). *Olearia* is represented in Victoria by 43 species, 22 of which are included on the FFG Act 1988 (DELWP 2021, VicFlora 2025). Various authors have worked within several sections and complexes of *Olearia s.s.* (e.g. Hawke 2005, Messina *et al.* 2013, 2014), highlighting the difficulty non-botanists may have in accurately identifying species and indicating that further investigation is required to ascertain taxonomic delimitations. Although hybridisation has been reported in *Olearia* (e.g. Heenan 2005), there is little information on its frequency, the conditions that promote hybridity, or whether there are negative or positive consequences. Hybridisation has not been confirmed genetically in Australian taxa but morphological intergrades considered likely to be hybrids have been observed for various Australian species (e.g. *O. rugosa* (F.Muell. ex W.Archer) Hutch. subsp. *rugosa* and *O. lirata* (Sims) Hutch. in Victoria, and *O. phlogopapp*a and *O. lirata* in Tasmania (Messina *et al.* 2013; VicFlora 2025)). *Olearia rugosa* from southeastern Australia is comprised of five geographically disjunct subspecies. Three subspecies, endemic to Victoria, are narrowly distributed, often occurring in small, isolated populations (Messina *et al.* 2014). A fourth subspecies occurs only on Bass Strait islands and the fifth occurs mainly in Victoria but its range extends to southeastern NSW.

The entire ranges of two East Gippsland taxa, *O. rugosa* subsp. *angustifolia* Hawke ex Messina and *O. rugosa* subsp. *distalilobata* Hawke ex Messina, were affected by the Black Summer bushfires of 2019/2020. As a result, the two subspecies became post-bushfire targets for rapid assessment of survival and for the collection of seed for conservation purposes (Gallagher 2020, DCCEEW 2023). *Olearia rugosa* exhibits clear taxonomic delineation (see Messina *et al.* 2013) in contrast to the ambiguity observed in other congeners. Genetic diversity studies for the restricted subspecies of *O. rugosa* were proposed to develop *in situ* and *ex situ* conservation frameworks and to guide genetic rescue of small, isolated populations. Post-bushfire censuses found individuals exhibiting morphological intergrades in leaf shape and indumentum where *O. rugosa* subsp. *angustifolia* occurs with *O. lirata* in the western part of this subspecies' range, raising the possibility of hybridisation between these taxa. Confirming the genetic status of the intergrades would aid in ensuring that conservation seed collections comprise target taxa and not hybrid seed.

In this study, genetic data was used to directly benefit the conservation of at-risk subspecies of *O. rugosa*. Our primary aim was to examine genetic structure in two of the four Victorian subspecies, and to test for hybridisation in a population of *O. rugosa* subsp. *angustifolia* growing sympatrically with *O. lirata*, where morphological intermediates were observed. This information can be used to prioritise actions that preserve genetic diversity *in situ* and guide sampling to maximise the genetic diversity of target taxa in *ex situ* collections.

## Methods

### Species background

*Olearia rugosa* is confined largely to Victoria, growing as a spindly shrub to 2.5 m high, with green, wrinkled leaves that are tuberculate above and stellate-tomentose below, with white ray florets surrounding yellow or purplish disc florets. There are five morphologically and geographically discrete subspecies, four of which occur in Victoria. Of those, two were affected by the Black Summer bushfires (Figure 1).

*Olearia rugosa* subsp. *allenderae* (J.H.Willis) Hawke ex Messina is restricted to swampy areas or gully slopes in Wilsons Promontory National Park, Victoria, and is listed as Endangered (EN) under the FFG Act (VicFlora 2025). Leaves of this subspecies are elliptic, ovate or narrow-triangular with deeply serrate to crenate margins.

*Olearia rugosa* subsp. *angustifolia* is naturally rare and often forms quite small (<20 plants), localised populations near streams and swamps, or on sandy rises in near-coastal areas of the East Gippsland lowlands. This subspecies is distinguished by leaves that are more

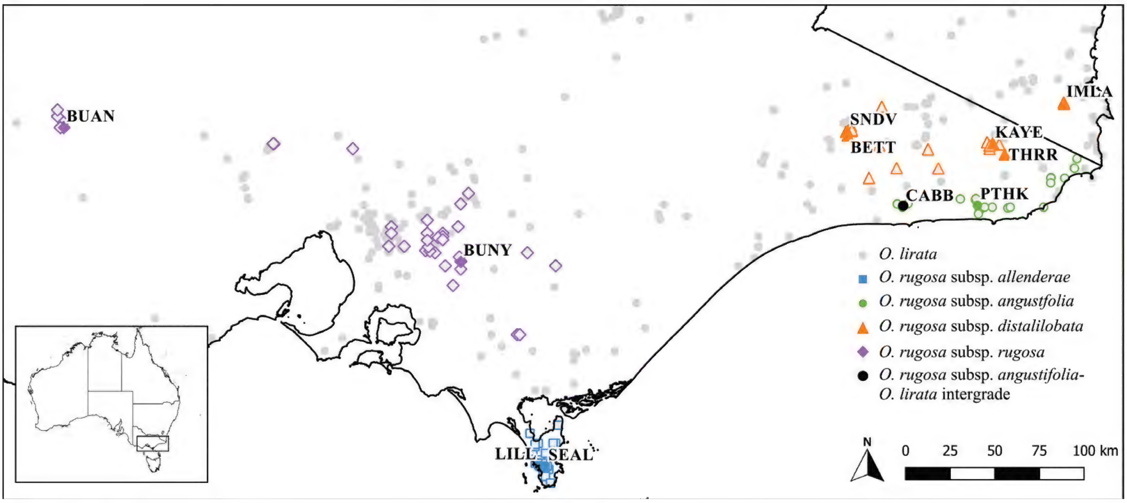
BUAN
BUNY
SNDV
BETT
KAYE
THRR
IMLA
CABB
PTHK
LILL
SEAL
● O. lirata
■ O. rugosa subsp. allenderae
● O. rugosa subsp. angustfolia
▲ O. rugosa subsp. distalilobata
◆ O. rugosa subsp. rugosa
● O. rugosa subsp. angustifolia-O. lirata intergrade
N
0 25 50 75 100 km

**Figure 1.** Four subspecies of *Olearia rugosa* and *O. lirata* in Victoria and southeast NSW with hollow symbols showing the overall distribution of each according to herbarium collections (AVH 2025) and filled symbols indicating locations sampled for this study. Colours are given in legend. Population codes are provided in Table 1, with CABB showing the location of CABBA, *O. rugosa* subsp. *angustifolia*, CABBL *O. lirata* and CABBU morphological intergrade collections.

or less linear with serrate to crenate margins and it is a candidate for FFG nomination given its geographic and demographic restriction. With the exception of Cabbage Tree Creek, all known locations in Croajingalong National Park were affected by the Black Summer bushfire. During post-bushfire surveys for this study, morphological intergrades were observed between *Olearia rugosa* subsp. *angustifolia* and sympatric *O. lirata* at Cabbage Tree Creek.

*Olearia rugosa* subsp. *distalilobata* occurs on rocky gully slopes of montane forest in remote areas from the Snowy River through to Mt Imlay in southeast NSW. This species is distinguished by its elliptic to oblong or obovate leaves with subentire or distally crenate margins. Eight populations were originally known and several factors, including logging, are likely to have caused local extinction at three locations in the last 30 years. This subspecies is listed as Endangered (EN) under the EPBC Act (1999) and five locations were affected by the Black Summer bushfire (DELWP 2022).

*Olearia rugosa* subsp. *intermedia* Messina is restricted to islands in Bass Strait and northeastern Tasmania. As the species epithet suggests, this species is characterised by a somewhat intermediate leaf shape (VicFlora 2025).

Finally, *Olearia rugosa* subsp. *rugosa* is distinguished by leaves that are ovate, elliptic or suborbicular with margins lobed throughout. It is the most widespread subspecies, occurring in wet to damp forest on southern slopes of the Dividing Range from Mount Macedon to Drouin, with isolated records from Mount Buangor and the Strzelecki Ranges. Possible hybrids and morphological intergrades with the widespread species *Olearia lirata* (Sims) Hutch. have been observed occasionally where these two species co-occur in the Beenak – Warburton area to the northeast of Melbourne (VicFlora 2025). *Olearia lirata* is a highly plastic congener that can occur sympatrically with all subspecies of *O. rugosa* from locations near sea-level to subalpine areas, in moist forest and scrub throughout southeastern Australia.

The four Victorian subspecies and one population of *O. lirata* were sampled. The two subspecies affected by the Black Summer bushfires, *Olearia rugosa* subsp. *angustifolia* and *O. rugosa* subsp. *distalilobata,* were the focus of this work and were sampled across their ranges. Two populations each were sampled for *O. rugosa* subsp. *allenderae* (highly restricted) and subsp. *rugosa* (more widespread) to assess genetic boundaries between subspecies. *Olearia rugosa* subsp. *intermedia* was not included in this study due to the significant geographic disjunction from the Victorian subspecies.

## Sampling

Field surveys targeted locations of *Olearia rugosa* subsp. *distalilobata* and subsp. *angustifolia* that were prioritised for action by the Department of Climate Change, Energy, the Environment and Water (DCCEEW 2023). In total, 11 locations were sampled for DNA analysis (Figure 1; Table 1) based on National Herbarium

**Table 1. Location and sampling details for *Olearia* used in this study including the dataset the samples were used in. Populations marked with an asterisk were affected by widespread bushfires in 2019/2020 and all plants sampled at these locations were seedlings up to two years old. Adult plants were sampled at all other locations.**

| Taxon | Location | Code | # samples | used in dataset |
|---|---|---|---|---|
| *O. rugosa* subsp. *rugosa* | Buangor | BUAN | 5 | 1 |
| *O. rugosa* subsp. *rugosa* | Bunyip | BUNY | 5 | 1 |
| *O. rugosa* subsp. *angustifolia* | Point Hicks | PTHK* | 5 | 1, 2 |
| *O. rugosa* subsp. *angustifolia* | Cabbage Tree Creek | CABBA | 5 | 1, 2 |
| *O. lirata* | | CABBL | 5 | 1, 2 |
| morphological intergrade | | CABBU | 16 | 1, 2 |
| *O. rugosa* subsp. *distalilobata* | Snowy Divide Track | SNDV* | 8 | 1 |
| *O. rugosa* subsp. *distalilobata* | Tulloch Ard | BETT* | 5 | 1 |
| *O. rugosa* subsp. *distalilobata* | Thurra River | THRR | 5 | 1 |
| *O. rugosa* subsp. *distalilobata* | Mt Kaye | KAYE* | 5 | 1 |
| *O. rugosa* subsp. *distalilobata* | Mt Imlay | IMLA* | 5 | 1 |
| *O. rugosa* subsp. *allenderae* | Lilly Pilly Track | LILL | 5 | 1 |
| *O. rugosa* subsp. *allenderae* | Sealers Cove Track | SEAL | 9 | 1 |

of Victoria specimens or Atlas of Living Australia observation records (Australasian Virtual Herbarium 2022) with the Cabbage Tree Creek location (CABB) comprising three separate collections. Five plants were sampled for each taxon at each location unless stated otherwise. In all locations, samples were gathered from plants believed to be separate individuals spaced at least 10 m apart across the geographic extent of the given population. Populations marked with an asterisk (Table 1) were affected by widespread bushfires in 2019/2020 and all plants sampled at these locations were seedlings up to two years old. Adult plants were sampled at all other locations. For all populations, a representative herbarium sample was collected for the National Herbarium of Victoria.

*Olearia rugosa* subsp. *distalilobata* was collected from four locations in Victoria (THRR, SNDV, BETT and KAYE), and one location in NSW (IMLA). Several historic collection locations for this subspecies in Errinundra and Snowy River National Parks are considered extinct. No attempts were made to re-survey these locations (DCCEEW 2023).

*Olearia rugosa* subsp. *angustifolia* was collected from only two sites, PTHK and CABB. No plants were found at Miners Track and the status of plants at the following locations is unknown as sites have been inaccessible for several seasons due to damage from bushfires and flooding: Cicada Trail, Benedore River Mouth, and Spotted Dog Mine. At Cabbage Tree Creek (CABB), plants of intermediate leaf shape and indumentum morphology were observed intergrading from typical subsp. *angustifolia* through to typical *O. lirata* and were considered possible hybrids between the two taxa. Therefore, in addition to five samples collected from plants identified morphologically as pure subsp. *angustifolia* (CABBA), samples were collected from 16 plants that varied across a morphological gradient from subsp. *angustifolia* to *O. lirata* (CABBU). An additional five samples, considered to be pure *O. lirata* based on morphology, were collected approximately 50 m away from the putative hybrid population (CABBL). Morphology was broadly assigned in the field then verified under the microscope upon return from the field. In contrast, no intergrades were observed at PTHK despite the presence of both *O. rugosa* subsp. *angustifolia* and *O. lirata*.

Samples were included from two sites each of *O. rugosa* subsp. *allenderae* from Wilsons Promontory (LILL and SEAL) and *O. rugosa* subsp. *rugosa* from Buangor (BUAN) and Bunyip State Forest (BUNY).

## DNA extraction, amplification and sequencing

Silica-dried leaf samples (83 samples, 11 technical repeats = 9 samples including technical repeats for each taxon) were sent to the commercial genotyping service, Diversity Arrays Technology ('DArT', Canberra, Australia) for DNA extraction and DArTseq$^{\text{TM}}$ analysis, a reduced representation sequencing method (Kilian *et al.* 2012). DNA was extracted at DArT using the Nucleo Mag kit (Machery Nagel, Germany) on a Tecan 100 platform following the manufacturers protocols. Library preparation involved DNA digestion and ligation using methylation-sensitive restriction enzymes PstI and MseI and uniquely barcoded adaptors. Following PCR and quantification, the samples were standardised and pooled for sequencing in a single lane of an Illumina NovaSeq X+ sequencer. Sequences were processed using proprietary DArT analytical pipelines to filter poor quality sequences. Resultant sequences were used in DArTsoft14, DArT PL's proprietary SNP calling algorithms.

## Data analysis

The unfiltered SNP dataset received from DArT consisted of 69,329 SNPs across 93 samples, with one sample failing sequencing (*O. rugosa* subsp. *distalilobata* from THRR). Initial screening for clones (genetically indistinguishable samples) was made by separating the dataset into broad taxonomic groups and using Euclidean genetic distance calculations via the R packages dartR (Gruber *et al.* 2018) and Poppr (Kamvar *et al.* 2014, 2015). All analyses were conducted in R (R Core Team 2019) version 4.4.1 unless otherwise specified. A threshold to select clonal samples was calculated as the average value between technical repeat pairs plus/minus three standard deviations of the repeat pair values. Any sample pairs below these values in the genetic distance measure were considered clonal samples. A kinship analysis using PLINK method of moment (MoM) for the Identity-By-Descent (IBD), analysis in SNPRelate (snpgdsIBDMoM; Zheng *et al.* 2012) was used to verify monoclonal individuals. Clonal samples were identified in some but not all populations of *O. rugosa* subsp. *distalilobata* (detailed below) which hindered balanced calculation of heterozygosity statistics for populations, so they are not reported here. Technical replicates were removed from all further datasets and analyses.

Dataset 1 was used to initially visualise relationships among species, subspecies and populations and included 82 samples, including clonal samples, across all 11 locations sampled. To enhance the data quality yet retain SNPs across broader phylogenetic groups, Dataset 1 was filtered for secondaries, read depth (5-90), a reproducibility threshold of 0.95, loci call rate of 0.4, individual call rate of 0.45, filtered for Hamming distance to remove possible paralogues and monomorphic loci using the R package dartR (Gruber *et al.* 2018). One *O. rugosa* subsp. *rugosa* individual from BUNY was removed in these steps, with the resultant Dataset 1 comprised of 13,261 SNPs across 81 individuals. A phylogenetic network was constructed with the NeighborNet method using the R package RSplitsTree (ver. 0.1.0, see http://rdrr.io/github/ IVS-UZH/RSplitsTree/) a GENPOFAD distance metric suitable for SNP markers, calculated in the 'pofadinr' R package (Joly *et al.* 2015) and visualised in R using ggplot2 (ver. 3.4.2; Wickham 2016) and tanggle (ver. 1.0.0, see http:// rdrr.io/github/ KlausVigo/tanggle/). Unlike bifurcating trees, phylogenetic networks can capture complex evolutionary histories (e.g. reticulation events) and thus may be more informative when assessing relationships among closely related species as well as infraspecific relationships (Huson and Bryant 2006).

Dataset 2 was used to interrogate the morphological intergrade observed at the CABB location and included all samples from the CABB location including *O. lirata* samples from this location and the *O. rugosa* subsp. *angustifolia* from PTHK. The raw dataset of 36,708 SNPs across 31 individuals was filtered as described above with the loci call rate set at 0.8, individual call rate of 0.57 and MAF set to 3% to give final Dataset 2 composed of 3,910 SNPs and 31 individuals. A principal component analysis (PCA) implemented in the adegenet package (Jombart, 2008; Jombart & Ahmed, 2011) in R (R Core Team, 2019) was used to visualise introgression between taxa across the Cabbage Tree Creek population. A Bayesian analysis implemented in STRUCTURE v. 2.3.4 (Pritchard *et al.* 2000) was used to identify K genetic clusters in the dataset without any information regarding taxon assignment or geographic proximity. The upper K limit was set to five on the assumption that there could be four potential genetic groupings. Ten independent runs were undertaken for each K value with a burn-in of 100,000 and 200,000 MCMC iterations. The R package pophelper (Francis 2017) and ggplot2 (Wickham 2016) were used to visualise results and select the most probable K based on the ΔK metric (Evanno *et al.* 2005).

The *gl.fixed.diff* function was utilised on Dataset 2 in dartR to export the top 200 loci (by call rate) exhibiting a fixed difference between the putative parental taxa using dartR package for use in a hybrid analysis (Gruber *et al.* 2018). Putative parent taxa in CABB were assigned based on morphological identification as either *z0* or *z1*, with samples from PTHK assigned *s*, pure origin *O. rugosa* subsp. *angustifolia* that were not to be considered part of the mixture relevant to the analysis. NewHybrids Version 1.1 beta (Anderson & Thompson 2002; Anderson 2003) was used to estimate the posterior probability (range 0–1) that individuals fall into pure parental, $F_1$ hybrids, $F_2$ hybrids or first-generation backcrosses based on the genotype frequency classes of the 200 selected loci, the Jeffreys Prior for θ and 10,000 burnin with 90,000 MCMC sweeps. The results from this analysis were used to assign a probability that each sample from CABB was either a parent taxon (*O. rugosa* subsp. *angustifolia*, *O. lirata*), hybrid taxon ($F_1$ or $F_2$ *O. rugosa* subsp. *angustifolia* x *O. lirata*) or a backcrossed hybrid taxon with either parent.

## Results

The raw data from DArTSeq contained 93 samples and 68,672 loci. The clonal analysis found genetically indistinguishable samples within several *O. rugosa* subsp. *distalilobata* populations, five samples within IMLA, two samples within KAYE and three samples within THRR. No other clones were identified within any of the taxonomic group datasets. After filtering as outlined above, each of the final SNP data sets in the analysis were the following dimensions: Dataset 1 contained 13,261 SNPs and 81 individuals across 11 locations and Dataset 2 contained 3,910 SNPs and 31 individuals across two locations.

## Species structure

The NeighborNet phylogenetic network from Dataset 1 visually displays the morphological intergrade observed at the CABB location with individuals identified as *O. rugosa* subsp. *angustifolia* and *O. rugosa* subsp. *angustifolia* x *O. lirata* hybrids and *O. lirata* from this location in one cluster (Figure 2). Two pairs of populations, BETT and SNDV (*O. rugosa* subsp. *distililobata*) and SEAL and LILL (*O. rugosa* subsp. *allenderae*), are connected at the base of the branches reflecting the historical genetic reticulation occurring due to the close geographic proximity of each pair. All other populations of each subspecies were clearly differentiated with many populations, including IMLA and BUAN, forming monophyletic clades on long branches showing little/no reticulation with neighbouring populations of the same subspecies.

## Hybridisation within Cabbage Tree Creek

The PCA based on Dataset 2 provides higher resolution of the CABB population with axes 1 and 2 accounting for 28.49% of the variation in the dataset. The species are separated across axis 1 with *O. lirata* (CABBL; squares) in the negative space and *O. rugosa* subsp. *angustifolia* from CABBA (circles) and PTHK (triangles) in the positive space (Figure 3a). The morphological intergrade

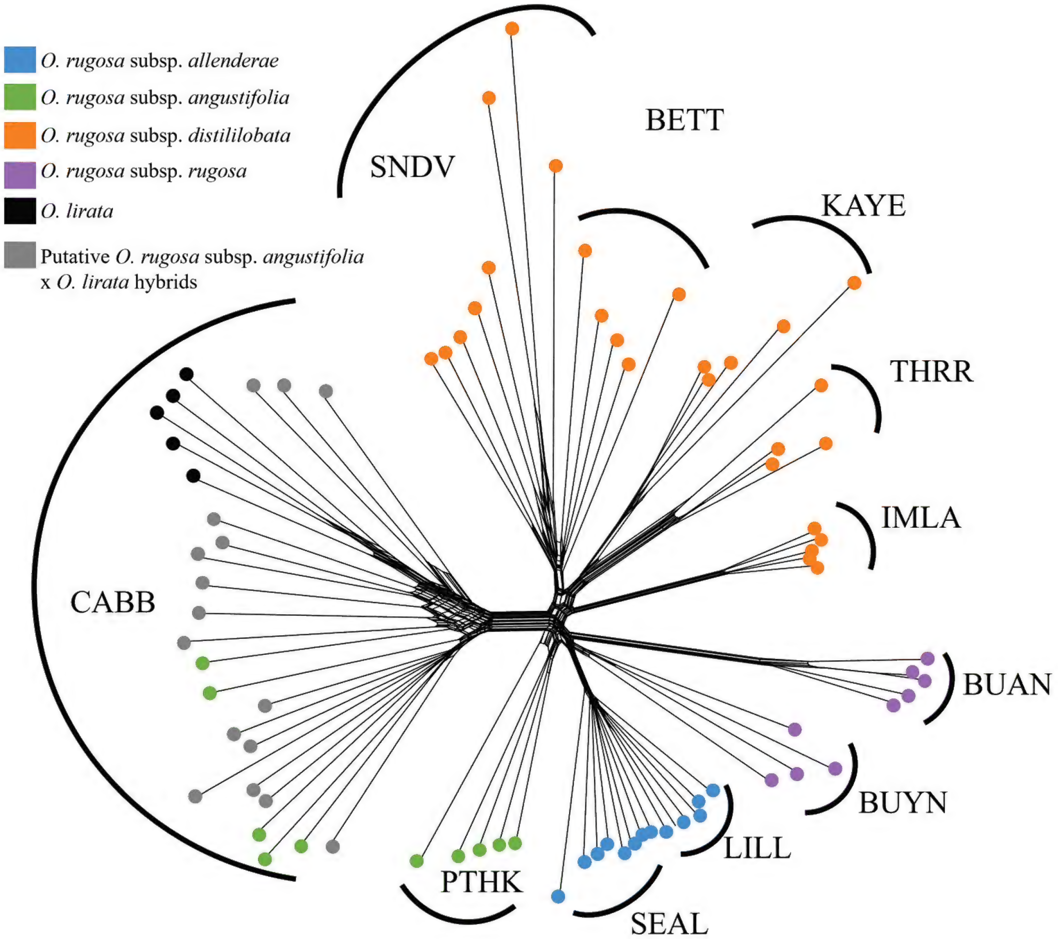
O. rugosa subsp. allenderae
O. rugosa subsp. angustifolia
O. rugosa subsp. distililobata
O. rugosa subsp. rugosa
O. lirata
Putative O. rugosa subsp. angustifolia x O. lirata hybrids
SNDV
BETT
KAYE
THRR
IMLA
CABB
BUAN
BUYN
LILL
SEAL
PTHK

**Figure 2.** Phylogenetic network based on GENPOFAD distance 13,261 SNPs and 81 individuals in *Olearia* Dataset 1 labelled by their population code and sample name, coloured by species as identified when collected. Black = *O. lirata*, olive green = *O. rugosa* subsp. *angustifolia*, grey = *O. lirata* × *O. rugosa* subsp. *angustifolia* morphological intergrade, orange = *O. rugosa* subsp. *distalilobata*, blue = *O. rugosa* subsp. *allenderae*, purple = *O. rugosa* subsp. *rugosa*.

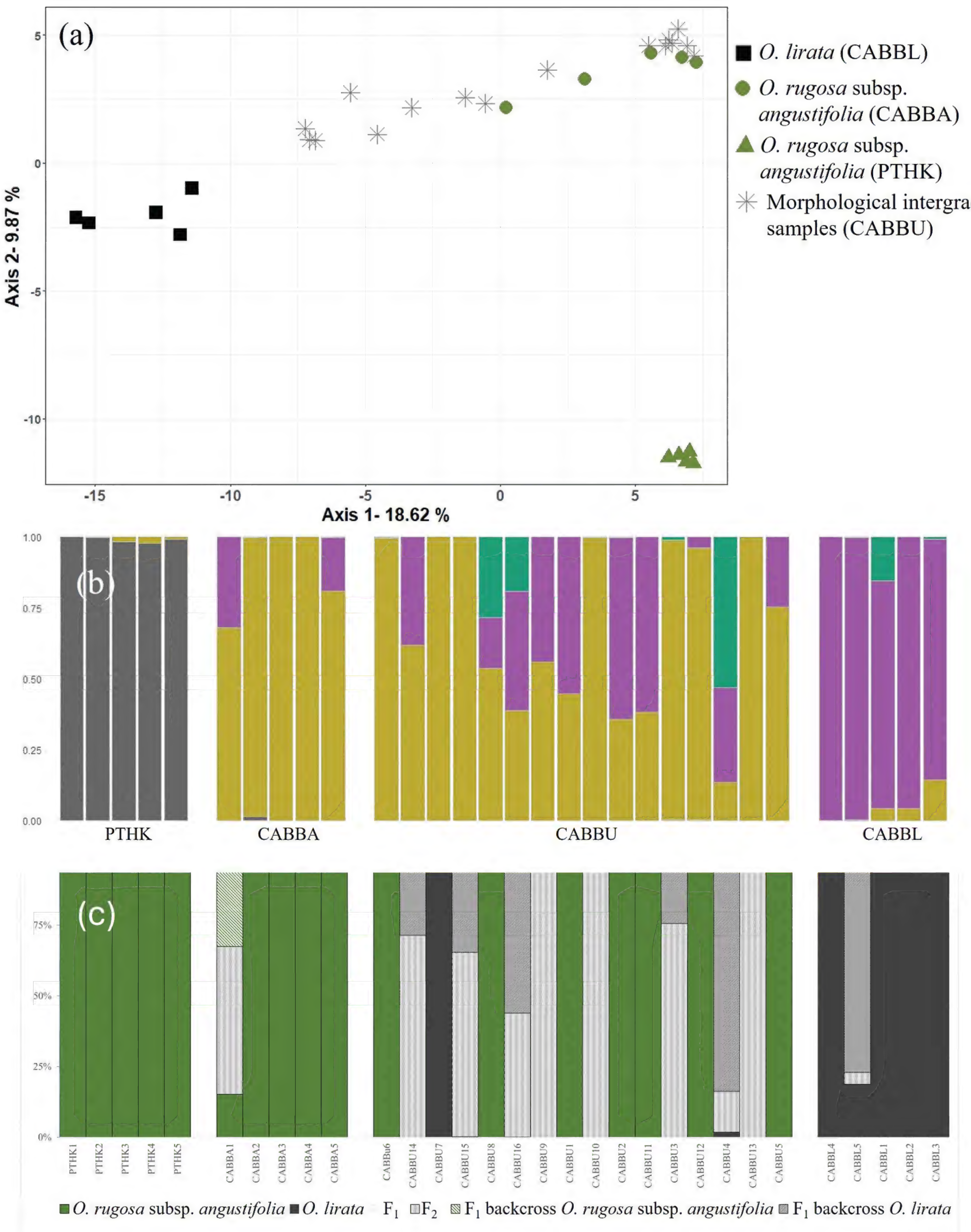

**Figure 3.** Results of genomic analysis of 31 *Olearia* individuals including *O. rugosa* subsp. *angustifolia*, *O. lirata* and morphological intergrades between these taxa from the CABB and PTHK locations based on Dataset 2 (3,910 SNPs). (a) first and second axis of the principal component analysis; individual samples are coloured by species and shapes indicate population of origin as detailed in legend. (b) STRUCTURE results displaying barchart of individual Q-membership proportions to each of K=4 clusters, with individuals clustered by population. Population labels apply to the chart displayed in (c). (c) The plot of the posterior probabilities of the genotype class estimated with NewHybrids with the proportion of each individual assigned to either pure species, F$_1$, F$_2$ hybrids or F$_1$-backcross. Individual labels below (c) apply to the chart displayed in (b).

samples from CABB (stars) placed intermediately (Figure 3a). Some *O. rugosa* subsp. *angustifolia* samples are placed towards the intermediate space and some of the morphological intergrade samples are placed closer to *O. rugosa* subsp. *angustifolia,* potentially suggesting backcrossing to this parent taxon. The *O. rugosa* subsp. *angustifolia* populations PTHK and CABB are separated across axis 2.

The Evanno ΔK method identified K=4 genetic clusters from the Structure analysis (Figure 3b). The morphologically typical *O. rugosa* subsp. *angustifolia* samples from the PTHK location were primarily composed of cluster 1 (grey). Cluster 2 primarily aligned with the *O. lirata* individuals from CABBL (purple). The *O. rugosa* subsp. *angustifolia* individuals from CABBA were aligned with a third cluster (yellow) although two individuals from this population showed admixture with cluster 2 (Figure 3b). The morphological intergrade individuals (CABBU) had several individuals that were either entirely made up of the third cluster (5) or primarily this cluster with a very small amount of admixture (2), five were admixed primarily cluster 3 with a portion assigned to cluster 2, while three others exhibited admixture between clusters 2, 3 and 4.

The results of the New Hybrids analysis provide further resolution on the admixture present at the CABB location and for the most part is consistent with the Structure results (Figure 3c). Four of the five *O. rugosa* subsp. *angustifolia* individuals (CABBA) were identified as pure species while one individual had similar probability assignment as an $F_2$ hybrid or a $F_1$-backcross with *O. rugosa* subsp. *angustifolia* (Figure 3c; Table 2). The *O. lirata* samples (CABBL) had a similar pattern with four individuals identified as pure *O. lirata* and one with a high probability of a $F_1$-backcross with *O. lirata*. The morphological intergrade samples (CABBU) ranged from a high probability of pure *O. rugosa* subsp. *angustifolia* (7) and pure *O. lirata* (1), to $F_2$ individuals (3), with the remaining five individuals $F_1$-backcrossed with *O. lirata*. One notable incongruence with the Structure results was the assignment of CABBU7 as pure *O. lirata* in the NewHybrids analysis while in the Structure results, this individual was assigned to cluster 3 (CABBA group). Of additional interest is an absence of $F_1$ hybrids. Of the 26 individuals from the CABB location (CABBA, CABBU, CABBL), only 14 had a field identification that was consistent with the identification from genomic analysis (Table 2).

## Discussion

The genus *Olearia* is one of the largest Asteraceae genera and this work adds to the number of conservation genetic studies made of threatened *Olearia* species (Smith *et al.* 2004, Barnaud & Houliston 2010) including those in the genomics era (Blyth *et al.* 2021). This genomic work has characterised the distinctiveness of populations within four of the five subspecies of *O. rugosa*, including *O. rugosa* subsp. *distalilobata,* listed under the federal EPBC Act, and *O. rugosa* subsp. *angustifolia,* listed under the Victorian FFG Act. Most populations assessed in this work were small and isolated, as such the barriers to connectivity between populations are evidenced in the lack of reticulation between populations in the NeighborNet analysis. A hybrid swarm, defined here as a morphologically variable group of plants consisting of parental species, hybrids and crosses among them, that was quantified in one population of *O. rugosa* subsp. *angustifolia* highlights the permeable nature of species boundaries and raises complications for conservation listings and *ex situ* conservation collections.

### Genetic structure and diversity

The structure of the phylogenetic network reflects the disjunct range of *O. rugosa*. Some populations of *O. rugosa* subsp. *angustifolia* and *O. rugosa* subsp. *distalilobata* are separated by more than 30 km. Even if continuous forest exists, gene flow would be limited between populations of each subspecies, resulting in genetic drift between populations over time. Similar patterns have been found for other threatened *Olearia* species (Smith *et al.* 2004, Blyth *et al.* 2021) and other endangered Asteraceae species (Rodger *et al.* 2021). Fire tolerance is not well understood for subspecies of *O. rugosa* and may have differing demographic and, consequently, genetic impacts depending on whether a species is a resprouter or obligate seeder. Obligate seeders are susceptible to local population extinctions when fire intervals are shorter than the time needed to re-establish seedbanks (Whelan 2002, Clarke *et al.* 2015) which could lead to a dramatic loss of genetic diversity. Resprouting enables the standing numbers of a species to rebound quickly, avoiding the loss of genetic diversity and a reliance on a seedbank. However, sexual reproduction is important as it enables the production of genetically variable seed and novel genotypes for landscape persistence, essential for longer term evolution and adaptation to changing environments (Bürger 1999). Little is known of the comparative phenology, reproductive biology and pollinators of the

**Table 2. *Olearia* individuals in Dataset 2 with the degree of hybridisation observed by morphological identification in the field and posterior probabilities of the genotype class estimated by NewHybrids.**

| Individual | Morphological ID | NewHybrids Result |
|---|---|---|
| **Point Hicks (PTHK)** | | |
| PTHK1 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| PTHK2 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| PTHK3 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| PTHK4 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| PTHK5 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| **Cabbage Tree Creek (CABB)** | | |
| CABBA1 | *Olearia rugosa* subsp. *angustifolia* | 50% $F_2$, 30% $F_1$ backcross *angustifolia*, 20% *angustifolia* |
| CABBA2 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBA3 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBA4 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBA5 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU1 | *Olearia lirata* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU2 | Hybrid | *Olearia rugosa* subsp. *angustifolia* |
| CABBU3 | Hybrid | 80% $F_2$, 20% $F_1$ backcross *O. lirata* |
| CABBU4 | *Olearia lirata* | 80% $F_1$ backcross *O. lirata*, 20% $F_2$ |
| CABBU5 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU6 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU7 | *Olearia lirata* | *Olearia lirata* |
| CABBU8 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU9 | *Olearia rugosa* subsp. *angustifolia* | $F_2$ |
| CABBU10 | *Olearia lirata* or hybrid | $F_2$ |
| CABBU11 | *Olearia lirata* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU12 | *Olearia rugosa* subsp. *angustifolia* | *Olearia rugosa* subsp. *angustifolia* |
| CABBU13 | *Olearia lirata* or hybrid | $F_2$ |
| CABBU14 | *Olearia lirata* | 70% $F_2$, 30% $F_1$ backcross *O. lirata* |
| CABBU15 | *Olearia lirata* or hybrid | 70% $F_2$, 30% $F_1$ backcross *O. lirata* |
| CABBU16 | *Olearia lirata* | 60% $F_1$ backcross *O. lirata*, 40% $F_2$ |
| CABBL1 | *Olearia lirata* | *Olearia lirata* |
| CABBL2 | *Olearia lirata* | *Olearia lirata* |
| CABBL3 | *Olearia lirata* | *Olearia lirata* |
| CABBL4 | *Olearia lirata* | *Olearia lirata* |
| CABBL5 | *Olearia lirata* | 80% $F_1$ backcross *O. lirata*, 20% *O. lirata* |

species. Plants are visited by a range of insects, which may or may not pollinate it, and there is no data on the species ability to self-pollinate.

A limitation of this study was the small numbers of samples collected from each location and an inability to compare genetic diversity results between populations due to the high levels of genetically indistinguishable samples in several *O. rugosa* subsp. *distalilobata* populations. Post-bushfire observations documented seedlings of *O. rugosa* subsp. *angustifolia,* while both seedlings and resprouting were documented in *O. rugosa* subsp. *distalilobata* (DELWP 2022). For the latter subspecies, resprouting was observed after high severity fire at Mt Imlay (IMLA) but the population suffered a decline in the number of live plants with only 42% of previously recorded plants observed to be resprouting (DCCEEW 2023). Thus, the identical genotypes observed at the IMLA, KAYE and THRR populations may be a result of sampling multi-stemmed resprouting individuals in the landscape. Additional detailed population demographic surveys and genetic studies, particularly at the IMLA location would assist in quantifying clonality and identifying levels of genetic diversity across the subspecies on the summit of this isolated mountain as genetic rescue may be an important component of conservation actions at this location. If an absence of multi-stemmed resprouting individuals is documented at the populations with a high number of clonal genotypes (IMLA, KAYE and THRR), investigations into mating systems may be necessary to eliminate the possibility of apomixis occurring within these populations as this would significantly alter the available genetic diversity at each site.

## Hybrid zone and implications for conservation

Increasingly genetically diverse *ex situ* conservation seed bank collections are playing strong roles in the conservation of—and as a germplasm source for—the restoration of species after events that may dramatically affect remnant populations (IUCN/SSC 2014, Martyn Yenson *et al.* 2021). During post-bushfire surveys for *O. rugosa* subsp. *angustifolia,* morphological intergrades between *O. rugosa* subsp. *angustifolia* and the more common *O. lirata* were observed at the CABB location. Some incongruence was noted between the results of the Structure and NewHybrids analyses. Structure detects admixture by gene flow between populations over time and for this study was based on the full dataset of 3,910 SNPs across all 31 individuals, while NewHybrids uses a subset of 200 SNPs to identify the probability that an individual is classified as either a pure parent, $F_1$, $F_2$ or backcross taxon. While the latter analysis can be sensitive to sample sizes and is limited to identifying specific hybrid classes, it is still useful in identifying cryptic hybrids that may not be morphologically distinguishable. The combined PCA, Structure and NewHybrids analyses identified that there is indeed an *O. rugosa* subsp. *angustifolia* × *O. lirata* hybrid swarm that occurs at CABB. At this point in time, it is unknown if hybridisation at CABB has been ongoing or was a rare event triggered by an environmental change. However, the sampling in this work did not identify any $F_1$ hybrids present in the CABB location, which could be either because they were not sampled or because $F_1$ hybrid plants have died out as $F_2$ hybrids ($F_1 \times F_1$ crosses) have been recruited in the population. A level of hybrid fertility is suggested by the presence of $F_2$ hybrids and $F_1$ backcrosses to one or the other parental species, suggesting hybridisation has been occurring here at least for several generations. The CABB location was not affected by the Black Summer fire and is an intersection between a lowland forest and a damp forest vegetation community which has a tolerable fire interval of greater than 25 years and has thus been excluded from contemporary fuel management programs (Cheal 2010). Therefore, it is entirely possible that putative hybrids identified in this study are from a chance opportunity where the boundary between the two forest types that supports the parental forms have met and the resultant putative hybrids have variable fitness in comparison to the parents (Harrison & Rand 1989). No other hybrid zones were identified visually at post-bushfire survey locations of *O. rugosa* subsp. *angustifolia* (PTHK and at the Miners Track location, which was not included in this study) despite *O. lirata* being recorded at PTHK.

While attempts were made to identify 'pure' individuals using morphology and keys during and after field collecting trips, the data presented here found that only 14 of the 26 field identifications assigned across a spectrum from pure *O. rugosa* subsp. *angustifolia* through hybrids to pure *O. lirata* were consistent with the hybrid identification gained from genomic analysis. It is therefore preferable for *ex situ* germplasm conservation collections to target alternative *O. rugosa* subsp. *angustifolia* populations to ensure that hybrid germplasm is not unknowingly stored or used in potential population enhancements. This highlights the morphological complexities that can arise in hybrid swarms. In many cases, only early generation hybrids are likely to be recognised morphologically because later generation hybrids and backcrosses resembling one or both parents will be identified as such (Abbott 2017, Gawrońska *et al.* 2023). Consequently, *O. rugosa* subsp. *angustifolia* × *O. lirata* hybrid zones comprising backcrosses may not have been recognised at other wild sites based on morphological analysis alone. Additional surveys and sampling from other *O. rugosa* subsp. *angustifolia* locations when accessible would assist in quantifying how often *O. rugosa* subsp. *angustifolia* and *O. lirata* occur sympatrically, whether morphological intergrades are present and the extent of hybridisation when the two are present. It would be useful to expand such work to include other subspecies of *O. rugosa* given that possible hybrids and morphological intergrades have been observed occasionally where *O. rugosa* subsp. *rugosa* and *O. lirata* co-occur in the Beenak – Warburton area to the northeast of Melbourne (VicFlora 2025). Examining breeding system, seed set and pollen viability across all morphological intergrades may help understand the evolutionary history and future of such populations.

A valuable outcome of this study was that surveys struggled to locate many historical populations of *O. rugosa* subsp. *angustifolia*. Being a hybrid swarm, CABB is possibly composed of very few 'pure' *O. rugosa* subsp. *angustifolia* individuals. Based on the relatively narrow range, the disjunction between populations, small population sizes, the likely reduction in both the number and size of populations, as well as the ongoing threat of growing along roadsides, this subspecies is worthy of consideration for assessment against IUCN criteria and would likely meet Endangered given the similarity to *O. rugosa* subsp. *distalilobata*. The IUCN assessment of this subspecies would need to take into consideration the fact that the size of the 'pure' *O. rugosa* subsp. *angustifolia* population at CABB is potentially only half of what is observable by morphology. An additional impediment to a rapid listing would be the need to survey at very isolated historical locations (VicFlora 2025), as well as at sites such as Miners Track where the species was initially found immediately post bushfire but not re-located thereafter. While the coastal lowland habitat is a mosaic of vegetation types with minimal land use change, several decades have passed since the species was previously recorded at some locations and the long-term survival of the species post-bushfire is unknown.

Hybridisation is a 'double-edged sword' for closely related species or subspecies which interbreed frequently, particularly if one taxon is considered rarer or has a competitive advantage over the other. Genetic swamping and introgression could lead to the local extinction of one or the other taxa or, for highly localised taxa, the extinction of entire lineages. On the flip side, the beneficial impacts of hybridisation include the provision of increased genetic diversity and negation of inbreeding depression, as well as driving the evolution of new, reproductively isolated species through homoploid hybrid speciation where there is no change in chromosome number, or allopolyploid speciation where increasing ploidy levels may improve or restore hybrid fertility (Schley *et al.* 2022; Todesco *et al.* 2016). Furthermore, ecological and genetic studies as well as quantification of ploidy within the various entities of the *O. rugosa* subsp. *angustifolia* hybrid swarm in the CABB population would be valuable for elucidating the taxon's conservation value from an evolutionary point of view. 'Stable' hybrid entities that maintain distinct hybrid morphology are afforded protection under the Commonwealth EPBC Act and the Victorian FFG 1988. However, under both Acts, there are no specified genetic or morphological criteria that must be satisfied for a hybrid entity to be deemed stable.

The Victorian Conservation Seedbank (VCS) holds seed collections for *O. rugosa* subsp. *angustifolia* from the PTHK location and for *O. rugosa* subsp. *distalilobata* from the BETT location. Additional collections of *O. rugosa* subsp. *distalilobata* from the KAYE and THRR locations and of *O. rugosa* subsp. *angustifolia* from any other population of this subspecies would enhance the representation of genetic diversity held *ex situ* given the genetic differentiation identified between locations assessed in this study. For collection at locations such as

CABB, where *O. rugosa* subsp. *angustifolia* and *O. lirata* grow sympatrically, caution should be given to the conservation value of the seed given the potential for hybrid seed. *Olearia rugosa* subsp. *distalilobata* can be propagated reliably from cuttings and therefore this may be an alternative for *ex situ* conservation of the species if care is taken to capture a diverse group of genotypes (McDougall *et al*. 2023). Forest fire interval planning should allow time for plants to reach maturity so that seed may accumulate in the soil seedbank to maintain genetic diversity over time.

Conservation of threatened species faces many challenges and, in the case of *Olearia rugosa* subspecies, the potential for hybridisation can complicate efforts to preserve taxa (Fitzpatrick *et al.* 2015), especially where hybridisation may be facilitated by human-mediated changes (Ottenburghs 2021). Combined with further genetic divergence leading to speciation of subspecies (Garmendia *et al.* 2022), this poses a conundrum in terms of deciding what to protect, particularly when remnant populations are few and small, a challenge that is increasingly encountered when making conservation decisions. If, as is the case here, there are few remaining populations, one is obliged to consider whether to engage in conservation actions aimed at preserving the "pure" genetics at the population, or to avoid interference with natural evolutionary processes (Genovart 2009). If the hybrid is outcompeting the parental genotypes and thrives over time to become a new taxon that is better suited to the conditions (e.g. Riesberg *et al.* 2003*)*, should resources be invested into preserving a moment in time along the evolutionary continuum? It may be advisable to develop a conservation strategy that incorporates hybrid populations as they may be a source of novel genetic diversity with potential adaptive advantages (Thompson *et al.* 2010). This could be facilitated by incorporating seed from hybrid populations as separate collections into the VCS. If population monitoring showed a high level of fitness, the stored seed could be used for restoration in similar habitats. By recognising the unique contributions of hybrids, conservation efforts might enhance resilience and promote long-term ecological stability.

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